[redacted]

Microbial Commercial Activity Notice

to the

U.S. Environmental Protection Agency

Office of Pollution Prevention and

Toxics Chemical Control Division

New Chemicals Notice Management Branch

TS#: BPS151

Date of Submission: 29 April 2015

Submitter: DSM Bio-based Products & Services

[redacted]

Contents

[redacted]

[redacted]

DSM

[redacted]

Certification of Information

I certify that to the best of my knowledge and belief:

1. The company named in this submission intends to manufacture, import, or process for a commercial purpose, other than in small quantities solely for research and development, the microorganism identified in this submission.

2. All information provided in the submission is complete and truthful as of the date of this submission.

3. I am including with this submission all test data in my possession or control and a description of all other data known to or reasonably ascertainable by me as required by 40 C.F.R. § 725.160 or § 725.260.

4. The company identified in this notice has remitted the fee of $2500.00 as specified at 40 CFR 700.45(b).

James La Marta, Ph.D

Date: 29 April 2015

James La Marta, Ph.D.

Senior Manager Regulatory Affairs

[redacted]

SUBSTANTIATION OF CONFIDENTIALITY FOR THE INFORMATION CLAIMED AS CONFIDENTIAL BUSINESS INFORMATION IN THIS MCAN

Section 1.3 of the MCAN provides a generic name for the microorganism pursuant to 40 C.F.R.§§ 725.80(a)(l) and 725.85(a)(3)(ii) (proposed as "*Saccharomyces cerevisiae* modified). Section 1.4 of the MCAN provides a generic category of use description pursuant to §§ 725.80(a)(2) and 725.88(b) (proposed as "ethanol production").

The following information is submitted in accordance with 40 C.F.R. § 725.94.

A. The nature of the Company's business is relatively unique in U.S. and international commerce. The technology is such that a competitor would be able to discern the production of the microorganism if the microorganism’s identity is publicly disclosed. A competitor, upon discovering this information, would have much less of an investment in research and development before being free to manufacture and sell or use the microorganism to our company's business detriment; such a disclosure is intolerable.

B. The Company has securely guarded information related to submitter identity, microorganism identity, process information, use, and internal company documents (excluding health and safety studies) so that the commercial utility of this information cannot be discovered by others. Only those with a need-to-know have access to this information.

C. Based on the foregoing, this information should be held confidential indefinitely, i.e., until this technology is obsolete, or until the microorganism is widely known as a result of competing research.

D. No advertising or promotional material, material safety data sheet or similar materials, professional or trade publications, other media, or local, state, or federal agency public files discloses the submitter identity in connection with the confidential identity of the microorganism or the information claimed as CBI in relation to its processing or use, or internal company documents (excluding health and safety studies). No Material Data Safety Sheet discloses this information. The Company's development of the microorganism has been held strictly confidential such that no competitor is aware that this microorganism is in use.

E. No federal, local, or state agency or court has public files disclosing the Company's identity, process, or referenced internal documents in connection with the microorganism. No Federal agency or court has ruled on the confidentiality of the microorganism.

F. Disclosure of submitter identity in connection with the confidential identity of the microorganism would allow competitors to devote fewer resources to research and development because they would be able to easily discern the microorganism and its commercial use.

G. Disclosure of the confidential identity of the microorganism would give competitors an advantage in knowing how to create the microorganism without the necessity of undergoing research and development to determine how best to create the microorganism. The manufacturer considers as highly confidential the identity of the gene used to modify the microorganism. This gene distinguishes the microorganism from more conventional strains and contributes new and useful performance properties to the microorganism. Disclosure would impart knowledge without any effort on the part of competitors.

H. Disclosure of process information and use and related internal company documents (excluding health and safety studies) would allow competitors to devote less resources to research and development and significantly reduce the commercialization time of competitors to create the microorganism.

I. Disclosure to the public of the information claimed confidential would allow a competitor to enter the market more easily because competitors have the facilities, personnel and expertise to produce the microorganism. Because the techniques for engineering the microorganism are generally familiar, the confidentiality of information related to the developed of the specific organism and its use must be maintained.

J. The strain that is the subject of this MCAN is covered by the following patents: [redacted]

[redacted]

[redacted] and national counterparts of these.

K. However, the microorganism is only one of many microorganisms that have been disclosed categorically. The identity of this microorganism should be treated as confidential because the patent does not disclose the microorganism's identity, *per se,* outside the context of many other micro-organism identities. Furthermore, the existence of such a patent does not necessarily indicate that this microorganism or any other member of the category for which patent claims have been made is in U.S. commerce.

L. The microorganism does not leave the site of production or testing in a form which is accessible to the public or its competitors. Unless the confidential information is disclosed, the cost to competitors to develop appropriate use conditions is several million dollars and three to five years. Confidential protection and secure handling impede product analysis by others.

M. The Company, pursuant to 40 C.F.R. § 725.92(b) and§ 725.95(e), claims as confidential references to microorganism identity and information that would facilitate the discovery of its identity in: (1)health and safety studies conducted by the submitter; and (2) published scientific journal articles submitted with the MCAN. Disclosure of microorganism identity would disclose confidential process and manufacture information that is unrelated to health and the environment. Disclosure of identity would reveal the nature of the modifications. Such disclosure would allow competitors to devote less resources to research and development because they would be able to more easily discern the modifications and commercial use. Furthermore, such disclosure would give competitors an advantage in knowing how to create the modifications without a commensurate investment in the research and development. Disclosure of these types of information would give competitors direct knowledge without any effort on their part. Less specific identity information is sufficient to interpret the references provided, because the results and conclusions of the researcher are fully disclosed by the articles.

[redacted]

1.1 Purpose

Pursuant to 40 C.F.R. § 725.250 DSM Bio-based Products & Services is filing this Microbial Commercial Activity Notice (MCAN) with the Environmental Protection Agency (EPA) for a modified transgenic microorganism engineered for use in ethanol production.

The recipient strain for the production organism is the well-characterized *Saccharomyces cerevisiae*. *S. cerevisiae* is an organism, which has an extensive history of safe use. In addition, the modifications to the recipient strain meet the conditions for introduced genetic material at 40 C.F.R. § 725.421 for the Tier I exemption. As EPA noted in its 1997 Final Risk Assessment for *S. cerevisiae* (p. 12) (EPA 1997), because the recipient microorganism was found by the agency to have little potential for adverse effects, "introduced genetic material meeting the specified criteria" of § 725.421 "would not likely significantly increase potential for adverse effects."

The production strain is intended for use in several dozen ethanol production facilities in the United States. It is not known whether all or some of these customer facilities will meet the containment criteria to qualify for the full Tier I exemption. In addition, it will be burdensome and time consuming for the Submitter and its customers to make this determination on a customer-by- customer basis. It is thought that the regulatory requirements of the Tier I exemption may make the commercial use of the production strain less attractive to customers. Thus, unless supported by a risk assessment, operational procedures and containment beyond what is normally employed at ethanol production facilities that currently use *S. cerevisiae* should not be imposed. Given the low hazard potential of the production strain, we respectfully conclude in this submission that current operating conditions at large-scale, conventional fermentation processes will not present an unreasonable risk in association with the use of the notified strain.

1.2 Contact Information

In accordance with 40 C.F.R. § 725.155(c), the following information is provided.

Submitter: DSM Bio-based Products and Services

Address: 1122 St. Charles Street

Elgin, IL 60120

[redacted]

Business Contact: Christian Koolloos

Business Manager, Bio-ethanol

+31(0)6-53 80 74 40

Technical Contact: James La Marta, Ph.D.

Senior Manager Regulatory Affairs

DSM Nutritional Products

45 Waterview Blvd.

Parsippany, NJ 07052

(973) 257-8347

[redacted]

2 Introduction

This introduction describes the construction overview of the "*Saccharomyces cerevisiae* modified". A number of genetic modifications in two parallel lines of strain construction have been brought about, as described in detail in section 2.1. The first line of strain construction has been designated the "[redacted] lineage" since genetically modified derivatives were derived from non-modified parental strain [redacted]; the second line is designated the "[redacted]" since the genetically modified derivative strains originate from a crossbreeding of non-modified parental strains from the [redacted] (*i.e.* [redacted]).

In the [redacted] lineage, strain [redacted], described in a previous [redacted] was repeatedly cultivated in batch cultures on lignocellulosic hydrolysates in order to select for spontaneous mutants which can grow faster on these mixed sugar substrates. Strain [redacted] was selected from a heterogeneous mixture of cells as one of the best performing strains. Subsequently, strain [redacted] was genetically modified. A heterologous arabinose transporter gene ([redacted] in some cases) was introduced in the [redacted], leading to an improved arabinose fermentation in the resulting transformants. Several transformants were screened and the best performing strain was designated [redacted]. Upon removal of the antibiotic resistance marker, a marker-free strain was isolated [redacted]. In the next step, strain [redacted] was transformed with two different constructs. In the first genetic modification, a copy of the [redacted] encoding an [redacted] was introduced in strain [redacted]. The [redacted] was targeted at the [redacted], thereby disrupting the latter. Several transformants were screened and the best performing strain was designated [redacted]. Upon marker removal, a colony was selected which had the same performance as [redacted] This strain was designated [redacted].

In the second genetic modification experiment, a [redacted] encoding an [redacted] thereby disrupting the latter. Several transformants were screened and the best performing strain was designated [redacted]. Upon marker removal, a colony was selected which had the same performance as [redacted]. This strain was designated [redacted].

The process of engineering the [redacted] lineage is depicted in the scheme below (Figure 1).

[redacted]

[redacted]

[redacted]

Figure 1: Schematic representation of the evolutionary engineering and genetic modification strategies leading to the isolation of strains [redacted]

For the [redacted], which was in constructed in parallel to the [redacted], identical modifications steps were conducted as in the [redacted]. Strain [redacted], described in a previous [redacted] was repeatedly cultivated in batch cultures on [redacted] in order to select for spontaneous mutants which can grow faster on these mixed sugar substrates. [redacted] was selected from a heterogeneous mixture of cells as one of the best performing strains. Subsequently, [redacted] was genetically modified. The aforementioned [redacted] in the

[redacted]

[redacted], leading to [redacted] in the resulting transformants. Several transformants were screened and the best performing strain was designated [redacted]. Upon removal of the antibiotic resistance marker, a marker-free strain was isolated [redacted]. In the next step, [redacted], was transformed with two different constructs. In the first genetic modification, a copy of the aforementioned [redacted] The [redacted], thereby disrupting the latter. Several transformants were screened and the best performing strain was designated [redacted]. Upon marker removal, a colony was selected which had the same performance as [redacted] This strain was designated [redacted] In the second genetic modification experiment, a copy of the aforementioned [redacted] thereby disrupting the latter. Several transformants were screened and the best performing strain was designated [redacted]. Upon marker removal, a colony was selected which had the same performance as [redacted]. This strain was designated [redacted]

The process of engineering the [redacted] is depicted in the scheme below (Figure 2).

[redacted]

[redacted]

[redacted]

Figure 2: Schematic representation of the evolutionary engineering and genetic modification strategies leading to the isolation of strains [redacted]

[redacted]

2.1 Proposed Generic Name

The explicit biological name of the microorganism is *Saccharomyces cerevisiae* strains containing modifications that add the ability to [redacted] needs at least the expression of the [redacted] is further improved by the introduction of [redacted]. Furthermore, [redacted] A generic name for this microorganism that is in accordance with 40 C.F.R. § 725.85 is "*Saccharomyces cerevisiae* modified."

This generic name protects the confidential identity of the [redacted] manufacturer of the MCAN microorganism considers as highly confidential the identity of the genes used to modify the microorganisms, [redacted] These genes distinguishes the MCAN microorganisms from more conventional *Saccharomyces cerevisiae* strains and contributes new and useful performance properties to these microorganisms. Nondisclosure of the specific genes used to modify the microorganisms is required to reduce the likelihood of a competitor manufacturing similar products, without investing time in conducting the necessary research and development required to develop such products. Throughout this document "fermenter" refers to the organism, and "fermentor" refers to the vessel.

2.2 Proposed Use Category and Generic Use Description

The confidential use category for the microorganism is [redacted] The submitter proposes the following generic use description: "ethanol production." This description protects from disclosure the confidential process and purpose of the manufacture while disclosing, with respect to exposure and release, the chemical to be produced.

[redacted]

3 Microorganism Identity Information

3.1 Recipient Strain Identification *Saccharomyces cerevisiae* strains

[redacted]

As mentioned previously in section 1.1, two lines of strain construction were pursued.

The parental strain for the [redacted] were cultivated repeatedly in batch cultures using different [redacted] and energy source. The composition of such [redacted] apart from fermentable sugars, such [redacted]). Such components, released by the breakdown of plant cell walls, prevent efficient conversion of sugar into ethanol and are therefore referred to as [redacted] However, upon sequential inoculation of fresh [redacted] original, unmodified *Saccharomyces cerevisiae* parental strain is also known as baker's yeast or brewer's yeast. The unmodified parental strains were selected from a yeast collection for one or more specific traits, relevant to the performance of the final modified strains in the final application: [redacted] and preserved or improved in the final

[redacted]

production strains derived from [redacted], either by originating from the unmodified parental strains or by introduction of genetic modifications.

The following unmodified parental strains were used as recipient for the genetic modified steps, as described in [redacted]:

[redacted]

[redacted]

There are no known pathogens associated with this taxonomic designation, thus, the designation is sufficient to distinguish the organisms from species that exhibit pathogenicity under the genus *Candida.*

Designation to the strain level is called for in section 725.155(d)(l) of the regulations.

The following additional information is provided. The abovementioned strains of *Saccharomyces cerevisiae* are genetically modified to have the ability to [redacted] Several modifications have been introduced in order to obtain further improved yeast strains, as depicted in Figure 1 and Figure 2, and described in further detail below, [redacted]
In general, modifications were designed to improve the property of [redacted] In order to obtain this result we [redacted] were transformed with one of two different constructs.

For the [redacted] thereby disrupting the latter. Several transformants were screened and the best performing strain was designated [redacted]. Upon [redacted], a colony was selected which had the same performance as [redacted]. This strain was designated [redacted]
For the other construct, [redacted], thereby disrupting the latter. Several transformants were screened and the best performing strain was designated [redacted] Upon marker removal, a colony was selected which had the same performance as [redacted]. This strain was designated [redacted].

[redacted]

For the [redacted] the one construct, a copy of the [redacted] thereby disrupting the latter. Several transformants were screened and the best performing strain was designated [redacted]. Upon marker removal, a colony was selected which had the same performance as [redacted]. This strain was designated [redacted] For the other construct, [redacted], thereby disrupting the latter. Several transformants were screened and the best performing strain was designated [redacted]. Upon marker removal, a colony was selected which had the same performance as [redacted]. This strain was designated [redacted]

[redacted]

[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]

[redacted]

[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]

[redacted]

[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]

[redacted]

[redacted]

[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]

It is of importance to note that the final result of the modifications will be [redacted]

3.2 Morphological Features of the New Microorganisms

Cells of [redacted] are unicellular entities that are globose (spherical) to ellipsoid in shape. Cell-size might be dependent on the ploidy. Cell size and morphology usually are very dependent on the way the cells are cultured.

The [redacted] might be flocculent, and will produce round, white to off-white colored colonies when grown on an agar plate. Flocculent strains will produce more rough edged colonies.

From a morphological perspective, [redacted] look like the majority of *Saccharomyces cerevisiae* cells/colonies. In other words, their appearance is not aberrant from other yeasts.

3.2.1 Physiological Features of New Microorganism

The genetic modifications introduced to *Saccharomyces cerevisiae* [redacted] will allow the final production strains, [redacted], respectively, to produce ethanol [redacted] more efficiently, as shown in Figure 3 below.

[redacted]

[redacted] *Saccharomyces cerevisiae* is enabled by the [redacted]

[redacted] Proprietary DSM data show that the [redacted]

Both [redacted] are present in the [redacted] which can be released by treating the [redacted] Upon treatment with [redacted]

In order to convert the fermentation [redacted], yet another gene was expressed. In [redacted] (see Figure 3).

[redacted]

[redacted]

[redacted]

[redacted]

Figure 3: Metabolic pathways involved in the production of ethanol from sugars [redacted]

3.2.2 Data by Which the Microorganism May be Uniquely Identified

Through the polymerase chain reaction (PCR) amplifications described in detail below, primers can be used to amplify specific regions only found in the production strain but not in non-modified strains. As reference, signatures of modifications made in the strain lineages [redacted]

In the scheme below it is shown, per modification, what specific fragments can be used as identifiers (signature). [redacted]

[redacted]

[redacted]

Selected PCR fragments for confirmation:

1) Signatures of modifications made in **all** strains [redacted]
   [redacted]

2) Signature of modifications in [redacted]
   [redacted]

3) Signature of modification in [redacted]
   [redacted]

[redacted]

3.2.3 PCR amplification of Signature [redacted]

One set of primers was designed to amplify the integration segment of the [redacted] As observed in the picture below, the set of [redacted] (at the correct height as observed by loading the 1kb Plus DNA ladder as reference in the right outer lane) in a candidate strain after marker removal in [redacted] and no product in a reference strain which did not contain the construct. In the negative control lane for the PCR procedure where the template for the PCR was demineralized water (H2O) the lane showed no product as well. [redacted]

[redacted]

1
2
3
L

[redacted]

[redacted]

[redacted]

[redacted]

3.2.4 PCR amplification Signature 2: [redacted]

One set of primers was designed to amplify the integration segment of the [redacted] As observed in the picture below, the set of primers [redacted] in a candidate strain [redacted] and no product in a reference strain which did not contain the construct. In the negative control lane for the PCR procedure where the template for the PCR was demineralized water ($H2O$) the lane showed no product as well. [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

3.2.5 PCR amplification Signature 3: [redacted]

One set of primers was designed to amplify the integration segment of the [redacted] in a candidate strain after [redacted] and no product in a reference strain which did not contain the construct. In the negative control lane for the PCR procedure where the template for the PCR was demineralized water (H_2O) the lane showed no product as well. [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

3.2.6 DNA sequences by which the Microorganism May be Uniquely Identified

For reference, the DNA sequences integrated into the genome of strains predecessors of [redacted]

[redacted]

[redacted]

In addition, in section 3.2.8, the sequence of a plasmid is shown which was used during the construction of the yeast strains. [redacted]

3.2.7 Modification [redacted]

The following DNA fragment is characteristic for genetic modification described herein as [redacted]

[redacted]

3.2.7.1 [redacted]

Integration construct, deletion of one locus of [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

3.2.8 [redacted]

Plasmid introduced [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

3.2.9 Modification Introduction [redacted]

The following DNA fragment is characteristic for genetic modification described herein as [redacted]

3.2.9.1 [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

3.2.10 Modification [redacted]

The following DNA fragment is characteristic for genetic modification described herein as [redacted]

3.2.10.1 [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

3.3 Plating Method and Data

The Submitter has provided a plating method in a previous [redacted] along with supporting data, to distinguish the production strain, which can grow on either [redacted] from an unmodified *Saccharomyces* strain that is unable to grow on these pentose sugars as the sole carbon source. Since the strains that are described in this MCAN are direct derivatives of the strains described previously the plating method is still valid to [redacted] from unmodified *Saccharomyces cerevisiae*. See Annex 2.

3.4 Genetic Construction of Strain [redacted]

3.4.1 The Taxonomy of the Donor Organisms

The intergeneric genes inserted into the production strains are the [redacted]

[redacted]

[redacted]

[redacted]

[redacted] in bacteria and some anaerobic fungi. The data substantiating the taxonomy of the donor organism is provided in the attached protein sequence analysis using a BLAST search comparing the [redacted]

[redacted]

In the process of breaking down biomass to free carbohydrates for their survival, filamentous fungi, such as [redacted]

Bacteria are able to convert [redacted] has a long standing tradition as model organism in the field of molecular biology [redacted]

[redacted]

[redacted]

3.4.2 Description of Traits for Which the Microorganisms Were Selected

Traits added or modified to select the commercial strains have been mentioned before in [redacted] and are still valid. These are summarized as follows:

- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]

[redacted]

[redacted]

[redacted]

Figure 4 [redacted]

The figure was copied from [redacted]

[redacted]

[redacted]

- [redacted]
- [redacted]
- [redacted]

[redacted]

[redacted]

- [redacted]

[redacted]

- [redacted]

3.4.3 Detailed Description of the Genetic Construction

All transformations with the constructs described below were carried out as described in [redacted]

Additional information on the construct as related to the markers is provided in **Section 4.2** of this MCAN.

For the modifications made previously in direct predecessors in [redacted]

To select for integrations of constructs dominant resistance markers were introduced and removed again after successful integration.

3.4.3.1 Integration of [redacted]

[redacted]

Fragments are made via conventional, textbook, molecular biology techniques. When needed constructs are cloned into plasmids and [redacted]

Upon introduction of these [redacted] of competent yeast cells, recombination will take place between the DNA sequences that share homology with each other, [redacted] Recombination typically occurs only in the order defined by the connectors, as indicated in the figure above.

[redacted] with the abovementioned fragments and selected on rich agar medium supplemented with [redacted]

[redacted]

[redacted]. Upon transformation connector sequences between the different construct parts would allow for correct assembly of the introduced genetic material in yeast at the right locus (see figure above). [redacted]

3.4.3.1.2 [redacted]

[redacted] were transformed with [redacted] Correct transformants were selected on rich agar medium supplemented with [redacted]. The [redacted] was constitutively activated upon transformation in the strains, selected colonies were immediately cultured without [redacted] were grown on rich growth medium (Yeast extract and phytone-containing media) [redacted]. From this culture new colonies were selected that [redacted] These colonies were streaked on different agar media to verify that [redacted] Diagnostic PCR amplifications were conducted additionally to verify that hallmark constructs were [redacted].

[redacted]

3.4.3.2 Integration of [redacted]

3.4.3.2.1 [redacted]

[redacted]

In the case of [redacted] (see figure above):

- [redacted]
- [redacted]
- [redacted]
- [redacted]

Fragments are made via conventional, textbook, molecular biology techniques. [redacted]

Upon introduction of [redacted] recombination will take place between the DNA sequences that share homology with each other, [redacted] Recombination typically occurs only in the order defined by the connectors, as indicated in the figure above.

[redacted]

[redacted] with the above mentioned construct parts and selected on rich agar medium supplemented with [redacted] for integration of the [redacted]. Upon transformation connector sequences between the different construct parts would allow for correct assembly of the introduced genetic material in yeast at the right locus (see figure above). [redacted]

3.4.3.2.2 [redacted]

Similarly as during the [redacted] The same procedure was maintained for these derived strains as described in section 3.2.8. Correct transformants were selected on rich agar medium [redacted] Selected colonies were grown on rich growth medium [redacted]. From this culture new colonies were selected that lost [redacted] These colonies were streaked on different agar media [redacted]. Diagnostic PCR amplifications were conducted additionally to verify that hallmark constructs were retained and marker loss did occur. [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

- [redacted]
- [redacted]
- [redacted]
- [redacted]

DNA fragments are made via conventional, textbook, molecular biology techniques. [redacted]

Upon introduction of [redacted], recombination will take place between the DNA sequences that share homology with each other, [redacted] Recombination typically occurs only in the order defined by the connectors, as indicated in the figure above.

[redacted]

[redacted] were transformed with the above mentioned DNA fragments and selected on rich agar medium [redacted] Upon transformation connector sequences between the different construct parts would allow for correct assembly of the introduced genetic material in yeast at the right locus (see figure below). [redacted]

3.4.3.3.2 [redacted]

Similarly as during the [redacted] The same procedure was maintained for these derived strains as described in section 3.4.3.1.2. Correct transformants were selected on rich agar medium [redacted]. Selected colonies were grown on rich growth [redacted]. From this culture new colonies were selected that [redacted] These colonies were streaked on different agar media to [redacted] Diagnostic PCR amplifications were conducted additionally to verify that hallmark constructs were retained and marker loss did occur. [redacted]

[redacted]

4 Phenotypic and Ecological Characteristics

4.1 Phenotype

The strain phenotype request is understood to refer to the expression of the genes of the organisms as well as the influence of environmental factors and random variation.

The unique characteristics that differentiates [redacted]. To obtain this, the strains have [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

- [redacted]

[redacted]

[redacted]

[redacted]

4.2 Antibiotic Resistance

The commercial strains do not contain any ARM (antibiotic resistance marker) genes. Antibiotic resistance markers were used in the development of parental strains but were later removed. The strains are naturally susceptible to antibiotics and to anti-fungals.

A PubMed search did not produce any articles related to resistance to antibiotics when [redacted]

[redacted]

Since the inserted genetic elements in this case do not appear to possess any intrinsic hazard potential, data are being provided for the species in general based on the rationale that the gene modification to the organism was not shown through a literature search to produce an effect or yield different results from the parental strain. For this reason, we believe it is appropriate to use the parental strain, *S. cerevisiae*, as a surrogate strain for gathering information and assessing the effect of the modified strain on antibiotic resistance and to tolerance to metals and pesticides. [redacted]

[redacted]

[redacted]. A null result is expected because resistance to antibiotics is not normally anticipated.

Based on the absence of demonstrated adverse effects for the parental strain and for the inserted intergeneric sequence, it is reasonable to conclude that the modified strain is not expected to be any different from other well-known *S. cerevisiae* strains commonly found in nature.

4.2.1 [redacted]

[redacted]

During the [redacted]

For the strains in the present MCAN in no case were plasmids introduced containing the [redacted].

In a [redacted] on predecessor strains we have checked thoroughly for the absence of [redacted] in the production strain by showing we cannot amplify the [redacted]. We have checked again for absence of the plasmid DNA in the production strains by showing [redacted]

A set [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted] [redacted] [redacted]

[redacted] [redacted] [redacted]

[redacted] [redacted]

[redacted] [redacted]

[redacted] [redacted]

[redacted] [redacted]

[redacted] [redacted] [redacted]

The resulting PCR reactions were run on an agarose gel and visualized by Nancy-520 staining, as shown by the representative analysis below:

[redacted]

[redacted]

As shown above, representative production strains (lanes 3-6) do not contain the [redacted] since the corresponding fragments for both genes were not amplified (upper panel and middle panel). The genomic DNA

[redacted]

samples representing the production strains [redacted]

4.2.2 [redacted]

During the experiments, some constructs [redacted]

[redacted]

The successful [redacted]

One [redacted]

[redacted]

[redacted]

[redacted] [redacted]
[redacted] [redacted]
[redacted] [redacted]

[redacted]

[redacted] [redacted]

[redacted]

[redacted]

4.2.3 [redacted]

During the experiments, [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

4.2.4 [redacted]

During the experiments, some constructs [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

- [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

4.3 Capacity for Genetic Transfer Under Laboratory and Environmental Conditions

We understand this question to bear on the ability of the organism to achieve dispersal of its genetic material through transfer of the genetic material to organisms in the environment that do not contain the genetic material. Thus, it is our understanding of this question that inheritance through budding is not considered dispersal of genetic material in this sense.

It is respectfully submitted that the transfer capability of the production strain is low because certain key elements that lead to, or improve the potential for transfer are noticeably absent from the construct. As an initial matter, the production strain does not contain any extra chromosomal elements, such as plasmids. [redacted]

[redacted]

[redacted] In addition, the inserted genes are not transposable elements nor do they contain any lysogenic viruses.

Data presented on sporulation demonstrate that [redacted] do not enhance the yeast's capacity to disperse or interact relative to the wild type strain under laboratory conditions.

As described in [redacted] of the first step for a diploid yeast cell to mate is for the cell to become haploid through the process of sporulation. [redacted]

[redacted]

The conditions present in the natural environment would not favor nor promote the dispersal of the gene to other cells. With respect to environmental conditions that might select for dispersal of traits, [redacted] is not expected to alter the conditions required for, and conditions that limit, dispersal and modes of interaction. Based on the rationale that the inserted genetic elements in this case do not appear to possess any intrinsic hazard potential, and are not expected to produce an effect that distinguishes it from the behavior of the parental strain for this purpose, additional reports on the dispersion of S. cerevisiae are being relied on as surrogate data to determine the possible mechanism of dispersion for the modified strain.

References are provided in the reference Bauer et al., that evaluate *S. cerevisiae*, a member of the *Saccharomyces sensu stricto* species complex, which contains most of the yeast strains relevant to the fermentation industry and basic science, including the closely related species *S. paradoxus*. In a laboratory setting, S. cerevisiae can mate with *S. paradoxus* with relative ease, but mating in the natural environment occurs mainly between cells of the same species, even though the two species coexist in the same environment. Sneigowski *et al.* have shown that S. cerevisiae demonstrates a stronger own-species preference compared to *S. paradoxus*. Even if mating occurs between two different species of Saccharomyces, the resulting hybrid are frequently sexually sterile. When S. cerevisiae was mated to *S. paradoxus*, the resulting hybrids were sexually sterile.

[redacted]

Environment Canada's review of *S. cerevisiae* strain ECMoOI cites a three year field study by Valero *et al.* (2005) to track the spread and survival of industrial yeast strains in vineyards of North Portugal and South France. This study does indicate that commercial and naturally occurring strains behave similarly. As noted in the government's review, any notified strain released into the environment as a result of large scale manufacturing can be dispersed by wind, fauna, or run-off of surface water. As reported by Reuter *et al.* (Reuter et. al. 2007), insects play a role in dispersion. The paper reports that fruit flies feed on yeast, and the spores that are ingested remain viable and are excreted and can be recovered from fly feces, but vegetative cells have not been recovered from the feces. The chapter by M. Begon in "The Genetics and Biology of Drosophila" provides similar information on the role of yeast in fruit fly nutrition(Begon, 1982). This vector would likely not play a large role for dispersion of the modified strain, as it has been shown that the modified diploid strain does not undergo sporulation and as a result, does not produce spores unless it mates with a haploid cell of the opposite mating type first. Once the cell mates, it has the ability to undergo sporulation to produce spores. Therefore; since vegetative cells have not been recovered from insect feces, it is not expected that the modified strain, which would be in the vegetative state and not the spore state, would be dispersed through insect vectors to a degree greater than well-known S. cerevisiae strains commonly found in nature.

Based on the absence of demonstrated adverse effects for the parental strain and for the inserted intergeneric sequence, it is reasonable to conclude that the modified strain is not expected to have an impact that is different from other well-known S. cerevisiae strains used commercially and/or commonly found in nature.

4.4 Habitat, Geological Distribution and Source of the Recipient Microorganism

The habitat of the parental or recipient strain, S. *cerevisiae,* also known as budding yeast, bakers' yeast and brewers' yeast, is diverse such that it is geographically distributed throughout the world. The U.S. Environmental Protection Agency has described the geographic distribution of S. *cerevisiae,* as has Environment Canada (CEPA 1999). Liti *et al.* (2006) report on the reproductive isolation of *S. cerevisiae.* Liti *et. al.* were able to isolate *S. cerevisiae* from each continent. In terms of source, "*S. cerevisiae* is a normal inhabitant of soils and is widespread in nature."[2] *S. cerevisiae* is known to be "ubiquitous in nature,

[2] *!d.* at p. 4

[redacted]

being present in fruits and vegetables."[3] Wild strains have been isolated from mushroom fruiting bodies as well as oak tree-associated soils and fluxes.[4]

4.5 Survival and Dissemination Under Relevant Environmental Conditions

The introduced [redacted] do not enhance the ability of the strain to exist in habitats different than that of the parental strain [redacted] See Lee et. al. (1970).

The production strain typically reproduces asexually through budding. The organism can reproduce sexually through mating. The first step for a diploid yeast cell to mate is for the cell to become haploid through the process of sporulation. Sporulation is the process where-the diploid yeast cell that contains both mating type loci a(MATa) and α(MATα) will undergo meiosis giving rise to four haploid cells: two MATa haploid cells and two MATa haploid cells. Mating can only occur between MATa and MATα haploid cells. Mating will never occur between two diploid cells or two cells of the same mating type.

The ability of the commercial strains to mate in a laboratory setting does not necessarily translate to an ability to mate in the natural environment because of reproductive isolation, especially when it comes to the availability of haploid cells of the opposite mating type. Wild yeast strains have the ability to shift mating type thus creating a mating possibility within the micro-colony. Therefore haploid cells will hardly be found in the natural environment since diploids will overgrow and out compete the haploid cells. The cost of gene expression also decreases fitness so modified strains are expected to have a disadvantage in natural environments compared to not modified strains (The cost of gene expression underlies a fitness trade-off in yeast, Lang et al., 2009, PNAS vol 106, no 14, 5755-5760)

Because the introduction of [redacted] is not expected to impart any

---

[3] **U.S. Environmental Protection Agency (February 1997) "Final Risk Assessment of Saccharomyces cerevisiae,"** [last updated Sept. 24, 2007].

[4] Capriotti A (1954) Yeasts in some Netherlands soils. Antonie van Leeuwenhoek 21: 145-156; Capriotti A (1967) **Yeasts from U.S.A. soils. Archiv fur Mikrobiologie 57: 406---413; Naumov Gf, Naumova ES, Korhola M (1992) Genetic identification of natural** *Saccharomyces* **sensu stricto yeasts from Finland, Holland and Slovakia. Antonie van Leeuwenhoek 61: 237-243; Naumov GI, Naumova ES, Sniegowski PD (1998)** *Saccharomyces paradoxus* **and** *Saccharomyces cerevisiae* **are associated with exudates of** North American oaks. Canadian Journal of Microbiology 44: I 045-1050; Goddard MR, Burt A (1999) Recurrent **invasion and extinction of a selfish gene. Proceedings of the National Academy of Sciences 96: 13880-13885.**

[redacted]

growth, replication, or survival differences compared to the wild type parental strain, it is appropriate to examine the opportunities for survival and dissemination of the parental strain.

References are being provided for the generic commercial strains as surrogate data (Capriotti, A (1954)) as well as data for two of the subject strains to evaluate the viability of the commercial strains in the natural environment. These references evaluate *S cerevisiae,* a member of the *Saccharomyces sensu stricto* species complex, which contains most of the yeast strains relevant to the fermentation industry and basic science, including the closely related species *S. paradoxus.* In a laboratory setting, *S cerevisiae* can mate with *S. paradoxus* with relative ease, but mating in the natural environment occurs mainly between cells of the same species, even though the two species coexist in the same environment Further, *S. cerevisiae* demonstrated a stronger own-species preference compared to *S. paradoxus.* Even if mating occurs between two different species of *Saccharomyces,* the resulting hybrid are frequently sexually sterile (Grieg *et al.).* When *S. cerevisiae* was mated to *S. paradoxus,* the resulting hybrids were sexually sterile.

To obtain these and other references, we used the search terms *"Saccharomyces cerevisiae"* and environ* and condition and (survive* or grow* or reproduce*). The following additional studies provided in Capriotti, A (1954)specifically assessed survival and growth in the laboratory or greenhouse under conditions designed to simulate the environment and found no differences in the survival kinetics of modified and wild type strains. We did not locate any papers in which such differences were observed in yeast. In the studies we reviewed:

- Insertions designed to enhance the output of the yeast did not appear to be a condition that enhanced or detracted from growth and survival.
- Normal environmental conditions (room temperature, neutral pH) did not affect comparative growth and survival.
- No differences in growth and survival were observed under the following conditions: a simulated vineyard environment, a soil/water suspension, a growth medium/soil environment, wastewater, and soil with a water content of 7.2% and a pH of 6.5.
- The primary condition identified as necessary for growth of modified or unmodified yeast is a nutrient rich environment.

As reported by Sniegowski et. al. 2002, a condition necessary for growth of modified or unmodified yeast is a nutrient rich environment. From Sniegowski et.al. it would be reasonable to conclude that *S. cerevisiae* can survive in the environment, such as in fluxes or soil, and when enough nutrients are present, a colony could grow through budding. Because [redacted] do not enhance the modified strain's ability to survive and grow, we would likewise expect that the modified strain would be able to survive in fluxes and soils of broad-leaved trees and be able to grow if a sufficient nutrient supply was available.

[redacted]

Bauer et al. (2004) reports the results of greenhouse trials evaluating the release and viability of modified *S. cerevisiae*. The conditions in the greenhouse were designed to simulate a vineyard. Yeast populations were applied by spraying to grapes, leaves, stem and soil weekly for one year and the progress of the modified yeast was evaluated against blocks of plantings left untouched. The authors report that:

> *"Although a high concentration of yeast was sprayed, few S. cerevisiae strains could be isolated at any given time. The yeast population in the sprayed blocks was otherwise very similar to the one found on the control vines, indicating that the commercial or GM yeast did not affect the overall ecological balance of the micro-flora. Furthermore, no significant differences between the behavior of the genetically modified and the parental strains could be detected.*

In year two, the same pattern was observed, with no significant difference with regard to presence in the greenhouse vineyard or cell numbers, suggesting that "the GM yeasts did not benefit from any specific advantage in terms of overall fitness when released in the vineyard."

Fujimura *et al.* (1994) studied a genetically-engineered strain of *Saccharomyces cerevisiae* employed for the industrial production of the human coagulation Factor XIIIa (rhFXIIIa) in a survival study under simulated environmental conditions. The strains were introduced into natural soil/water suspension, into soil/medium suspension and into waste water. The homologous strain devoid of the recombinant plasmid and the homologous strain bearing the 2 microns-based vector plasmid without the rhFXIIIa-encoding DNA insert were compared. After intervals, samples of cell suspensions were taken and viable cell numbers were determined by plating on antibiotic- containing medium. No differences in survival rates could be detected for the plasmid-bearing and plasmid-less strains under the three environmental conditions tested (soil/water suspension, YEPD medium/soil, and wastewater), suggesting that the presence of plasmid does not confer selective advantages on the survival of the yeast cells. The authors conclude that, even after accidental release of the engineered yeast cells into the environment, elimination rates would be comparable to those for non-recombinant yeast strains. The study noted that excessive growth of fungi and bacteria may be a condition that inhibits the survival of yeast cells (p. 991) in soil. Soil and wastewater were noted as poor in nutrients for the growth of yeast cells as well (p. 993). Ando *et al.* (2005) evaluated several modified yeast strains, haploid and diploid in soil with a water content of 7.2% and a pH of 6.5 and compared with growth in sterile water conditions. In this study there was no significant difference in the survival of viable cells and DNA in the soil environment among the strains tested. The viable cell and DNA concentrations decreased in a similar, time-dependent manner in soil and the decrease rate of the modified yeast strain was significantly higher than the wild type in water. A potential,

[redacted]

additional condition not notified above but which was identified in this study as a factor in determining the number of viable cells is presence or absence of *ATH1* loci (in *ATH1* disruptants, trehalose accumulates and functions as a cryoprotectant under freezing conditions. Disruption of *ATH1* improves the freeze tolerance of commercial baker's yeast, such that *ATH1* disruptants are used commercially in frozen dough baking). The presence or absence of this loci in the notified organism has not been determined, and should not be viewed as a factor necessary for the evaluation of the notified organism based on a single reported study.

The published papers discuss pH, temperature and nutrient requirements. We did not locate any articles that contained specific discussions of salinity or oxygen conditions. Based on the absence of demonstrated conditional differences between wild type and modified yeasts, it is reasonable to conclude that the modified strains are not expected to behave differently from S. *cerevisiae* strains commonly found in nature. See **Annex 4** for the results of environmental survivability studies with [redacted]. See **Annex 5** for the results of environmental survivability studies with [redacted]. The final results indicated that there is no significant difference between the engineered strains and the corresponding wild-type *Saccharomyces cerevisiae* strains regarding survival capabilities in the environment.

4.5.1 Description of Method for Detecting the Microorganism in the Environment

Section 3.2 of the MCAN provides details concerning three methods for detecting the microorganism in the environment: PCR analysis, DNA sequencing, and a plating method.

4.6 Anticipated Biological Interactions with Target Organisms and Other Organisms

As shown in the MCAN for strain [redacted] the modified *Saccharomyces cerevisiae* had no effect on the germination or growth of grass or corn. The plants were chosen on the basis of ease of manipulation, material accessibility and presence in the host range. More specifically, these species of plants would be the most likely recipients of accidental exposure due to their close proximity of [redacted]. There is no reason why the modified *Saccharomyces cerevisiae* described in this MCAN would behave different from the strain [redacted]. Concerning other biological interactions:

- <u>Host range</u>: The range of host species or cell types which the commercial strains are able to infect or parasitize is expected to be no different than that for the parent strains that are typically used in [redacted] today. In this regard, no host is needed for the S. *cerevisiae* to survive.

[redacted]

- Target organism: The use of the commercial strains is [redacted]. The microorganisms are not designed to act upon a particular organism during the production process or otherwise.
- Competitors: [redacted]
- Prey: The typical commercial medium used for production is composed of corn steep liquor (as a nitrogen source), glucose (carbon source), lactose (carbon source), various minerals, phenyl acetic acid (as a precursor), and a buffer (such as calcium carbonate). The medium can either be sterilized prior to addition to the [redacted] The microorganisms are not designed to prey upon living organisms as a food source.
- Hosts: The use of the commercial strains is for ethanol production in a [redacted] The microorganisms are not designed to be a host or to infect or feed upon another living organism. The commercial strains are not of the type that exhibits parasitic behavior with grapevine *(Vilis vinifera* L.) plants, the parasitic behavior of certain strains being considered novel and associated only with certain strains of *S. cerevisiae* that exhibit filamentous forms. See **Annex 6** for data on a predecessor strain.
- Symbionts: The commercial strains are not designed to be an organism in a symbiotic relationship. They are not designed to serve as a host in which the presence of a smaller symbiont beneficiary would be present.
- Parasites: No significant interaction with parasites to report.
- Pathogens: No significant interaction with pathogens to report. The commercial strains are not designed to enhance any pathogen such as *Escherichia coli, Clostridium botulinum.*

4.7 Pathogenicity, Infectivity, Toxicity, Virulence

4.7.1 Nonhuman Pathogenicity

With regard to any genetic basis for pathogenity and toxigenicity of the inserted [redacted], a PubMed search using the terms [redacted] and tox*, pathogen*, and infect* does not return any articles demonstrating [redacted] pathogenic to non-human species or that this pathway would cause the organism to exhibit

[redacted]

toxigenicity. A null result was not unexpected since [redacted] has been used in industry and food for years (see [redacted]) without any adverse effect. There are no studies that the submitter could locate that would indicate the donor strain itself, [redacted] is pathogenic in non-humans. [redacted] where its likely role is to [redacted]

The non-human pathogenicity and toxigenicity of the inserted genes from [redacted] is commonly known and accepted. [redacted] Its safety was described extensively in a review by [redacted] The European Food Safety Authority granted [redacted] the QPS (Qualified Presumption of Safety) status (see reference EFSA QPS (2012).

Because the gene modifications to the organism were not shown through literature searches to be toxic or yield different toxicological results from the parental strain, surrogate information on the recipient strain is offered for the purpose of evaluating the anticipated behavior of the production strain.

With regard to non-human pathogenicity, as concluded by Environment Canada in its *Risk Assessment Summary Conducted Pursuant to the New Substances Notification Regulations (Organisms) (NSNR[o]) of the Canadian Environmental Protection Act, 1999, EAU- 288:Saccharomyces cerevisiae* strain *ECMoO1 (August 23, 2006)* "reports of *S. cerevisiae* pathogenicity to insects, birds, fish, animals, and plants in the available scientific literature are exceedingly rare." The Environment Canada risk assessment notes one reported case associating *S. cerevisiae* with chronic diarrhea in a dog (Milner et. al. 1997). We found no further results identifying non-human species infectivity, toxin production, conditions under which toxins are produced, or involvement of the micro-organism as an obligate or opportunistic pathogen. We did not locate any discussion of biota known to be susceptible to the microorganism, except for one paper that showed that certain strains of *S. cerevisiae*, especially strains isolated from fermenting Champagne wine must, can slow down growth or cause necrosis in young grapevine plantlets in a laboratory setting (See Gognies et. al 2001). This group demonstrated that a general yeast strain from the America Type Culture Collection show little effect on the growth of young grapevine plantlets compared to yeast strains isolated from Champagne wine must, and did not provide data on the ability of the yeast to act as a pathogen in the wild or on adult grapevine plants. We also did not locate any known toxicity resulting from by-products of the biodegradation pathways of the organism.

[redacted]

In EPA's Final Risk Assessment of *Saccharomyces cerevisiae* (February 1997), the agency discussed the ability of a fungus to impair the host's immune capabilities in connection to the anticipated effect on non-human species and concluded that *S. cerevisiae* is nonpathogenic. EPA states (p. 4):

The cell walls of most fungi have the capacity to impede the immune response of the host. In a study to determine the overall pathogenicity of a number of yeasts used in industrial processes, animals exposed to both high levels of S. cerevisiae and cortisone demonstrated a greater ability of the fungus to colonize compared with those animals treated with only the yeast. However, the animals suffered no ill-effects from exposure to S. cerevisiae (Holzschu et al., 1979). Therefore, this study suggests that even with the addition of high levels of an immunosuppressant agent, S. cerevisiae appears to be nonpathogenic.

The agency goes on to conclude (p. 9) that *"The organism is not a plant or animal pathogen. Despite the fact that S. cerevisiae is ubiquitous in nature, it has not been found to be associated with disease conditions in plants or animals. "*

4.7.2 Effects in Humans

No studies were located that would indicate the donor strain [redacted] is pathogenic to *humans.* A review of the CDC and LCDC websites did not yield any involvement *of* [redacted] in adverse health effects.

The QPS-status of [redacted], its long history of natural occurrence and safe use in a variety of food products as well as the large number of reports describing its safe use by human as summarized in a the review of de Vries et al. (de Vries et al., 2006) indicate the absence of pathogenicity of [redacted] for human.

Because the gene modifications to the organism were not shown through literature searches to be toxic or yield different toxicological results from the parental strain, surrogate information on the recipient strain is offered for the purpose of evaluating the anticipated behavior of the production strain.

The American Type Culture Collection (ATCC) has classified *S. cerevisiae* as a Biosafety Level (BL) 1 organism based upon the fact that the organism is not known to cause disease in healthy humans. A review of the U.S. Centers for Disease Control (CDC) and Canada's Laboratory Centre for Disease Control (LCDC) websites did not yield any involvement of *S. cerevisiae* in adverse health effects. A search using *"Saccharomyces cerevisiae"* and pathogen* did not turn up any studies that indicated that the strain contains pathogenic genes.

There are reports that *S. cerevisiae* is an opportunistic pathogen. A 2006 chapter by McCusker provides a list of *S. cerevisiae* infections described in the literature. The list includes infections in patients with AIDS; it does not identify which of the other patients were otherwise immunocompromised. A 2005 report by Munoz *et a/.* described three (3) ICU patients that had *S. cerevisiae* fungemia at Hospital General Universitario. As part of the report, the authors searched MEDLINE for reports of *S. cerevisiae* fungemia since 1966. Their search returned only fifty seven (57) additional reported cases. Since S. *cerevisiae* is commonly used in the biotechnology industry, Murphy and Kavanagh (1999) examined the potential pathogenicity of *S. cerevisiae.* They concluded that *S. cerevisiae* can be regarded as an opportunistic pathogen for the immunocompromised, but one of low virulence. Copies of these papers are provided in McCusker and others.

As EPA recognized in its Final Risk Assessment of *Saccharomyces cerevisiae* (February 1997) (p. 9), "(m)any scientists believe that under appropriate conditions any microorganism could serve as an opportunistic pathogen." The agency concluded that S. *cerevisiae* has an extensive history in food processing and neither it nor other closely related species "has been associated with pathogenicity toward humans or has been shown to have adverse effects on the environment" (p.2).

Specifically, with respect to human exposure, EPA concluded on p. 3 of the Final Risk Assessment that:

> *There are individuals who may ingest large quantities of* S. *cerevisiae every day, for example, people who take the yeast as part of a "health food" regimen. Therefore, studies were conducted to ascertain whether the ingestion of large numbers of these yeasts might result in either colonization, or colonization and secondary spread to other organs of the body. It was found that the installation of very large numbers of* S. *cerevisiae into the colons of animals would result in both colonization and passage of the yeasts to draining lymph nodes. It required up to* 10^{10} *S. cerevisiae in a single oral treatment to rats to achieve a detectable passage from the intestine to the lymph nodes (Wolochow et. al., 1961). The concentrations of S. cerevisiae required were well beyond those that would be encountered through normal human daily exposure.*

EPA concluded that: *"Saccharomyces, as a genus, present low risk to human health or the environment. Criteria used to differentiate between species are based on their ability to utilize specific carbohydrates without relevance to pathogenicity. Nonetheless, this risk assessment applies to those organisms that fall under the classical*

[redacted]

definition of S. *cerevisiae as described by van der Walt (I971)."* Because the production strain falls under the classical definition described by van der Walt (1971), it is respectfully submitted that the production strain may be deemed nonpathogenic to humans.

4.7.3 Virulence

The ability for an organism to be pathogenic is promoted by the presence of virulence factors, such as proteases, lipases, and toxins. Because the gene modifications to the organism were not shown through literature searches to be toxic or yield different toxicological results from the parental strain, surrogate information on the recipient strain is offered for the purpose of evaluating the anticipated behavior of the production strain. In this regard, EPA has concluded that *S. cerevisiae* is nonpathogenic to humans and the production strain should be considered likewise nonpathogenic. Specifically, EPA observes in its Final Risk Assessment (p. 4) that:

> *A number of individual virulence factors have been identified as being associated with the ability of yeasts to cause disease. The principal virulence factors associated with yeasts appear to be phospholipase A and lysophospholipase. It is believed that these enzymes enhance the ability of the yeast to adhere to the cell-wall surface and result in colonization as a first step in the infectious process. Nonpathogenic yeast had considerably lower phospholipase activities. Of a wide range of fungi assayed for phospholipase production,* S. *cerevisiae was found to have the lowest level of activity (Barrett-Bee eta/., 1985). Therefore, based on the phospholipase virulence factor* S. *cerevisiae is considered a non-pathogenic yeast.*

The Final Risk Assessment identifies the potential for *S. cerevisae* to be pathogenic toward other yeast. As EPA states on p. 3:

[redacted]

> *There have been no reports of isolates of S. cerevisiae that produce toxins against either humans or animals. However, S. cerevisiae has been shown to produce toxins against other yeasts. These toxins, termed "killer toxins", are proteins or glycoproteins produced by a range of yeasts. The yeasts have been genetically modified to alter activity and are used in industrial settings as a means of controlling contamination of fermentation systems by other yeasts (Sid et. al., 1988)*

EPA goes on to conclude on p. 4:

> *S. cerevisiae does not carry virulence factors to humans or animals. However, the species does carry linear, double-stranded plasmids, which can be transmitted to other Saccharomyces. These plasmids carry genes that encode the "killer toxins" discussed above {sic] can be transferred from one Saccharomyces to another. Therefore, gene constructs involving the incorporation of traits using these linear plasmids should be considered to be non-stable.*

In the present case, the production strain is not known to produce toxins against humans, animals, or other yeast. The production strain does not contain any extra chromosomal elements such as plasmids. The original strain did contain plasmids, but the plasmids were removed, with the genes originally contained in the plasmid constructs inserted into the genome by targeted insertion -in order to stably integrate the sequences. In addition, the inserted genes are not transposable elements nor do they contain any lysogenic viruses.

4.8 Immunologic Reactions

The potential allergenicity of the inserted heterologous gene protein products was evaluated following the guidelines developed by the FAO/WHO consultation ((FAO/WHO, 2001); (FAO/WHO, 2009)). Following these guidelines, the amino acid sequence of the introduced enzymes are compared with known allergens. Cross-reactivity between the

[redacted]

expressed protein and a known allergen has to be considered when there is either more than 35% identity in the amino acids sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty, or identity of short contiguous amino acids segments (i.e. at least 8 contiguous amino acids).

For the comparisons, the database AllergenOnline™ (available at http://www.allergenonline.org/) was used. The amino acid sequence comparison of [redacted] not show 35% or more overlap with known allergens using a window of 80 amino acids; exact matches of 8 amino acids or more were not observed. It can therefore be concluded that the introduced enzymes are not likely to produce allergenic or sensitization reactions.

Since the inserted genetic elements in this case do not appear to possess any intrinsic hazard potential, data are being provided for the species in general, based on the rationale that the gene modification to the organism was not shown through a literature search to be toxic or yield different toxicological results from the parental strain.

Baldo and Baker (1988) examined the results of skin prick tests and radioallergosorbent tests (RASTs) and found positive reactions to protein extracts from *S. cerevisiae* and purified enolase from *S. cerevisiae* in people with inhalant allergies to airborne fungi. The study emphasized that although the results demonstrate a high incidence of positive skin tests and RAST reactions in those subjects, it does not mean that if the subjects were exposed to the proteins, an allergic response would occur. The tests merely demonstrate that the subjects have antibodies against the proteins, but presence of an antibody does not equate to an allergic response.

A more recent study by Horner et al., (2008) examined the ability of commercially produce fungal enzyme extracts on IgE antibody reactivity by RAST, including *S. cerevisiae* enzymes. The paper did not examine the sensitivity of subjects to the fungal enzymes, supporting the conclusion that commercially produced enzyme extracts could be used as source material for clinical allergen testing .

No further studies examining the potential allergenicity of *S. cerevisiae* were found, nor any studies examining the sensitivity of allergic responses to *S. cerevisiae*, nor any studies examining worker exposure and allergy responses in the baking and ethanol industry. Therefore, exposure to the modified *S. cerevisiae* is not expected to elicit any allergic response to the workers exposed during ethanol production.

[redacted]

4.9 Action as a Vector for Pathogens

A PubMed search using the terms [redacted] and pathogen* and "vector" does not return any articles demonstrating [redacted] permits the production strain to act as a vector of pathogens. A **null** result was not unexpected since [redacted] has been used in industry and food for years **(**Bhosale et. al. 1996**)** without any adverse effect. There are no studies that the submitter could locate that would indicate the donor strain itself, [redacted] acts as a vector for pathogens. The non-human pathogenicity and toxigenicity of the inserted genes from [redacted] is commonly known and accepted. [redacted] Its safety was described extensively in a review by [redacted] The European Food Safety Authority granted [redacted] the QPS (Qualified Presumption of Safety) status (see [redacted]

Because the gene modifications to the organism were not shown through literature searches to yield different results from the parental strain, surrogate information on the recipient strain is offered for the purpose of evaluating the anticipated behavior of the production strain. Based on the information provided in EPA's February 1997 Final Risk Assessment and Section 4.7.3 of this MCAN, the production strain is not expected to be a vector for pathogens. The production strain is not expected to act as a vector of any pathogen such as *Escherichia coli* or *Clostridium botulinum*.

4.10 Anticipated Involvement in Biogeochemical or Biological Cycling Processes

For the strain to [redacted], the [redacted] which requires some kind of chemical, enzymatic, or heat treatment. As a result, the commercial strains are not anticipated to have an effect on or to mediate any biological cycling processes in a manner that would be different from the parental strain, except for the ability to use [redacted]

Similarly, as [redacted] do not exist in the environment, the addition of [redacted] to the modification is not expected to result in an effect on the biogeochemical cycle that would be different from the parental strain. Based on the rationale that the inserted genetic elements in this case do not appear to possess any intrinsic hazard potential, and are not expected to produce an effect that distinguishes it from the behavior of the parental strain, data are being provided for the parental species in general as a surrogate for

[redacted]

gathering information and assessing the modified strain's involvement in biological and biogeochemical cycles.

A search of the scientific literature using the search terms "*Saccharomyces cerevisiae*" and nutrient cycle terms such a "carbon cycle," "nitrogen cycle," "phosphorus cycle," and "sulfur cycle" bears out that *Saccharomyces cerevisiae* is not known to play a lead role. We located one article with relevance to assessing the potential role of the organism in mediating the sulfur cycle which describes the metabolism of sulfur into the sulfur amino acids in *Saccharomyces cerevisiae.* The review pertains to the biological sulfur cycle, which consists of: (I) degradation;(2) dissimilatory oxidation; (3) dissimilatory reduction; and (4) assimilatory reduction. Yeast, and all eukaryotic plants and microorganism carry out assimilatory reduction to metabolize sulfur. See Thomas and Surdin-Kerjan, 1997.

We conclude that *S. cerevisiae* does not abnormally influence these cycles and that the genetic changes in the modified strains do not change the strain' role in any way from *S. cerevisiae.*

Based on the absence of demonstrated adverse effects for the parental strain and for the inserted intergeneric sequence, it is reasonable to conclude that the modified strains are not expected to have an adverse effect on biological or biogeochemical cycle.

[redacted]

5 Byproducts during Manufacture, Processing, Use and Disposal of the Strain

The initial stage of yeast manufacture is the production of the inoculum which takes place in a laboratory setting. Under laboratory conditions, inefficient mixing can allow for slightly anaerobic conditions to exist. Under anaerobic conditions yeast produce ethanol rather than replicating. It is anticipated that only a small quantity of ethanol and other volatile compounds will be produced during inoculum growth.

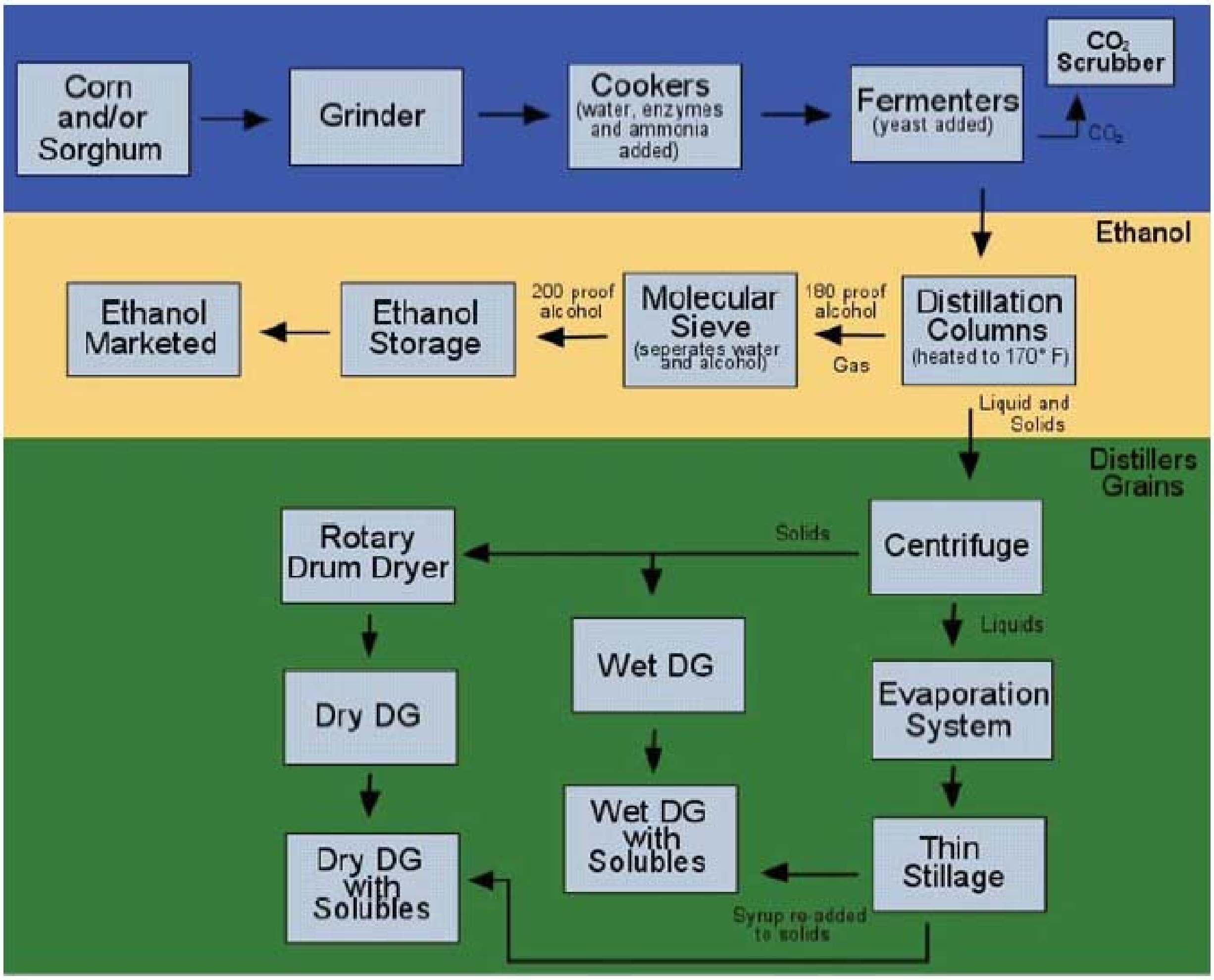
Based on EPA's AP-42 emission data (**EPA 1995**), approximately 80 to 90 percent of total VOC emissions is ethanol, and the remaining 10 to 20 percent consists of other alcohols and acetaldehyde. Acetaldehyde is a hazardous air pollutant as defined under Section 112 of the Clean Air Act. Volatile byproducts form as a result of either excess sugar present in the fermentor or an insufficient oxygen supply to it. Under these conditions, anaerobic fermentation occurs, breaking down the excess sugar into alcohols and carbon dioxide. When anaerobic fermentation occurs, 2 moles of ethanol and 2 moles of carbon dioxide are formed from one mole of glucose. Under anaerobic conditions, the ethanol yield is increased, and yeast yields are decreased. Therefore, in producing yeast, it is essential to suppress ethanol formation in the final fermentation stages by incremental feeding of the molasses mixture with sufficient oxygen to the fermentor.

During processing and use in ethanol production, expected byproducts are distillers dry grain (DG)composed of the solids remaining at the end of the fermentation (including inactivated biomass that has use as a high value animal feed ingredient). Carbon dioxide, plant oils, glycerol, lactic acid and acetic acid are additional byproducts of ethanol fermentation. Gluten is also a byproduct of certain fermentation processes. The DG solids byproduct production is diagrammed below in Figure #2.

In a second generation ethanol production facility it is anticipated that any residual cellulosic biomass and the spent yeast will be sent to an onsite energy production unit for use as a fuel. See figure #6

[redacted]

Figure #2 Distillers Grains Production Process



Kansas State University Agricultural Experiment Station 2009

[redacted]

5.1 Total Production Volume

The anticipated production volume for the first three years of operation are:

[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]

The submitter does not anticipate being the sole supplier of yeast to the bioethanol industry.

5.2 Commercial Formulation

The commercial product will be fluid bed dried, active modified yeast; commercially referred to as Active Dried Yeast (ADY).

[redacted]

5.3 Concentration of Modified Yeast in the Commercial Product

The commercial product will be approximately:

Appearance:	Tan, free flowing granules
Yeast dry matter	90 to 94 % w/w
Water	4 to 8 % w/w
Sorbitan monostearate (SPAN 60) emulsifying agent	2.0 % w/w
Total yeast count [redacted]	[redacted]

[redacted]

6 Use Information

6.1 Description of the Intended Category of Use

100% of the produced microorganism will go to industrial ethanol production The product is intended as a drop in replacement for yeast used currently in ethanol production, and projected increases in ethanol production volume that are currently anticipated based on publicly available estimates have been considered in the following analysis. The product data sheet is provided in Annex 9.

Based upon the maximum amount of yeast to be produced and the anticipated number of facilities that will utilize the modified yeast, the quantity of modified yeast per facility [redacted]

		[redacted]	[redacted]		[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	

Although the submitter plans to sell the modified yeast for use in as many ethanol plants as possible, the submitter does not anticipate being the sole supplier of yeast to the bioethanol industry and therefore believes that the volumes provided in section 5.1 are the most realistic estimate of the quantity of modified yeast that will be produced.

[redacted]

7 Worker Exposure and Environmental Release

The Submitter anticipates that there will be six ethanol plants in the US using the production strain in year three of production. Ethanol facilities are to be under the control of others, and the following representative assessment may not accurately represent every fermentation facility.

7.1 Sites Controlled by the Submitter

7.1.1 Production Facility

The modified *S. cerevisiae* will be grown, concentrated, dried and packaged at:

[redacted]

7.1.2 Process Overview

[redacted]

[redacted]

[redacted]

Figure #3 Modified *S. cerevisiae* Production Process Confidential Image

CONFIDENTIAL

[redacted]

[redacted]

7.1.3 Process Description for the Production of Active Dried Modified Yeast

[redacted]

[redacted]

[redacted].

7.1.4 Containment and Control Technologies

[redacted]

725.422 (a) Use a structure that is designed and operated to contain the new microorganism

[redacted]

[redacted]

[redacted]

[redacted]

725.422 (b) Control access to the structure

[redacted]

725.422 (c) Provide written, published, and implemented procedures for the safety of personnel and control of hygiene

[redacted]

[redacted]

[redacted]

725.422 (d) Use of inactivation procedures demonstrated and documented to be effective against the new microorganism contained in liquid and solid wastes prior to disposal of the wastes. The inactivation procedures must reduce viable microbial populations by at least 6 logs in liquid and solid wastes.

[redacted].

[redacted]

725.422 (e) Use features known to be effective in minimizing viable microbial populations in aerosols and exhaust gases release from the structure, and documented use of such features.

[redacted]

725.422 (f) Use systems for controlling dissemination of the new microorganism through other routes, and document use of such features

[redacted]

725.422 (g) Have in place emergency clean-up procedures

[redacted]

7.1.5 Worker Exposure and Environmental Release

[redacted]

725.155(h)iii Worker exposure and environmental release for Site Manufacturing of the Active Dry Modified Yeast

- [redacted]
- [redacted]
- [redacted]
  - [redacted] employees per shift, [redacted] each run max of 3 days.

[redacted]

- Fermentation: [redacted] employees [redacted] each run max of 4 day.
- Separation through Packaging: [redacted] employees per shift [redacted] each run max of 4 days

725.155(h)iv Release

[redacted]

725.155(h)v Transport of Active Dry Yeast from manufacturing facility

[redacted]

725.155(h)vi Procedures for disposal

[redacted]

7.2 Process Description of Sites Not Controlled by the Submitter

7.2.1 Identity of Sites where the Production Strain will be Processed and Used

[redacted]

[redacted]

7.2.2 Corn-based Ethanol Fermentation[5]

Today, U.S. ethanol is primarily produced from corn crops by dry-mill or wet-mill processing. Although wet-mill facilities were common in the industry's early days, dry-mill facilities now account for more than 80% of industry capacity. Between 2000 and 2007, the number of ethanol plants more than doubled and production capacity tripled in the United States. Most of the growth came from dry-mill plants because they are dedicated and optimized for the production of ethanol. Dry-mill plants are typically smaller than wet-mill plants and use less energy per gallon of ethanol produced. In both dry and wet-mill plants, the production of co-products consumes a third or more of total process energy. These co-products provide an important revenue source to ethanol producers.

Ethanol Facility Statistics www.icminc.com; per telephone conversation with company representative on September 24, 2010.

Average number of fermentors per facility: 4

Average fermentor gallon size: 500,000 gallons

Average gallons of ethanol produced each year: 50,000,000 gallons

Average number of batches per year per facility: 100 batches

7.2.3 Dry Mills

[5] Source: US Dept. of Energy. http://www.afdc.energy.gov/fuels/ethanol_production.html

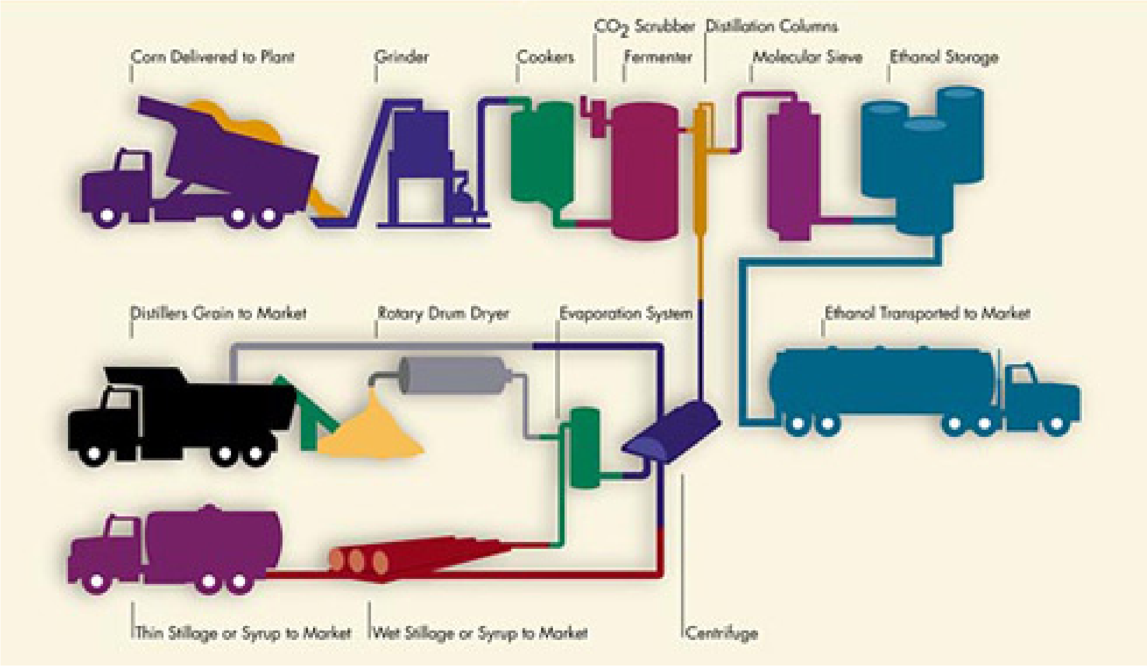
[redacted]

Dry-mill ethanol plants are optimized to produce ethanol with carbon dioxide (CO_2) and animal feed as co-products. In these facilities, the corn is ground into coarse flour. Next, water and enzymes are added, and the mixture is "cooked." Yeast is added, and the mixture is fermented.

This "mash" is sent to the distillation system and molecular sieves to remove the water to produce 200-proof ethanol. The ethanol is denatured (usually with gasoline) to make it unfit for human consumption and sent to ethanol storage tanks.

The solids and liquids remaining after distillation are generally recombined for sale as high- protein animal feed (known as wet distillers grains with solubles or WDGS). Some facilities also incorporate dryers to remove the moisture from the WDGS and to extend its shelf life. This dried co-product is called dried distillers grains with solubles (DDGS). The CO_2 co-product is commonly captured and marketed to the food processing industry for use in carbonated beverages or the production of dry ice.

Figure #4. Dry Mill Ethanol Process



Source: Renewable Fuels Association

Most dry-mill plants generate thermal energy (steam and hot air) on site by burning fossil fuels such as natural gas or coal. Electricity is typically purchased from a utility. One

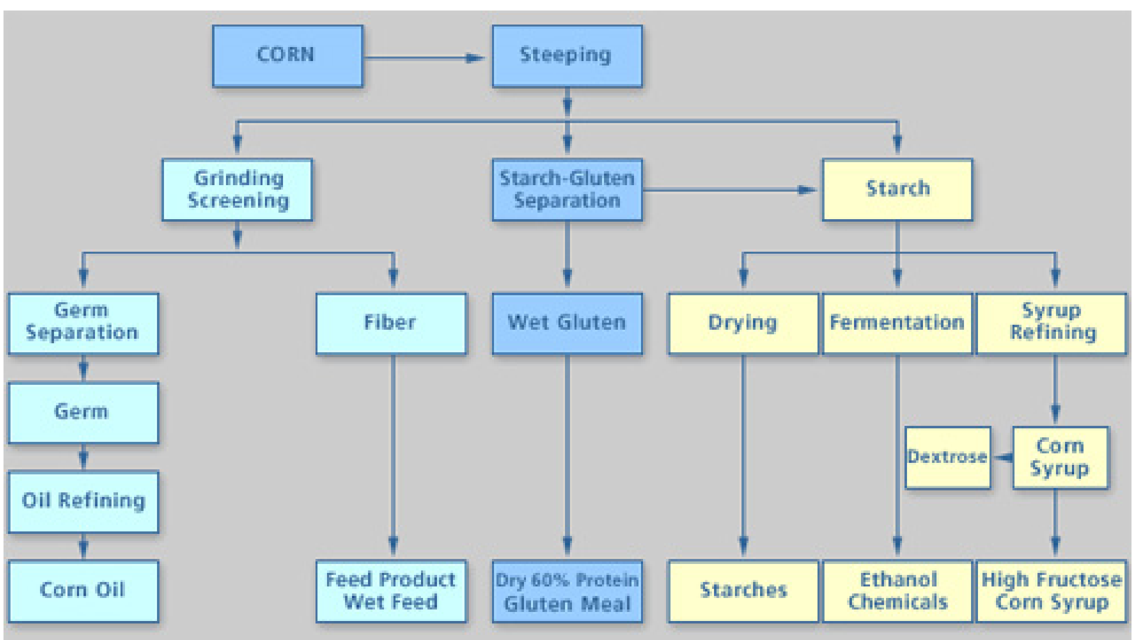
[redacted]

way to improve the efficiency of dry-mill plants is to use combined heat and power (CHP) systems. In a CHP system, thermal and electrical energy are generated together on site. According to EPA, CHP can reduce the energy used during ethanol production by I 0% to 25%.

7.2.4 Wet Mills

Wet-mill plants primarily produce com sweeteners, along with ethanol and several other co- products (such as corn oil, animal feed, and starch). In these mills, the first step is to soak the corn grain in hot water to separate the protein and starch. The product is then coarsely ground, and the germ is separated to be processed into corn oil. Next, the remaining slurry, which contains gluten, starch, and fiber, is finely ground and separated so the fiber can be blended into animal feed and the starch/gluten mixture can be further processed. The starch is then dried to make com starch or processed to produce sugars, corn syrup, and beverage sweeteners. The sugars are then fermented to produce ethanol. Most wet-mill plants produce their own thermal energy and electricity using CHP systems.

Figure #5. Wet Mill Process



Source: Renewable Fuels Association

[redacted]

7.2.5 Cellulosic Ethanol Production[6]

Ethanol can also be produced using cellulosic feedstocks. These are more difficult to break down into fermentable sugars than starch- and sugar-based feedstocks. As a result, the cellulosic biochemical conversion process requires additional steps (see diagram below). Two key steps are biomass pretreatment and cellulose hydrolysis. During pretreatment, the hemicellulose part of the biomass is broken down into simple sugars and removed for fermentation. During cellulose hydrolysis, the cellulose part of the biomass is broken down into the simple sugar glucose. There are two areas being explored to improve the efficiency and economics of the ethanol production process.

- **Cellulose hydrolysis.** The crystalline structure of cellulose makes it difficult to hydrolyze to simple sugars, ready for fermentation. Researchers are developing enzymes that work together to efficiently break down cellulose.

- [redacted]

The commercial strains that is the subject of this MCAN is being developed to address the second area above.

A. [6] Source: US Department of Energy.

B. http://www.afdc.energy.gov/fuels/ethanol_production.html

[redacted]

The following process flow diagram shows the basic steps in production of ethanol from cellulosic biomass. While cellulosic ethanol is not yet commercial in the U.S., it has been demonstrated by several groups, and commercial facilities are being built in North America. Note that there are a variety of options for pretreatment and other steps in the process and that some specific technologies combine two or all three of the hydrolysis and fermentation steps within the shaded box.

Figure #6 Cellulosic Ethanol Production

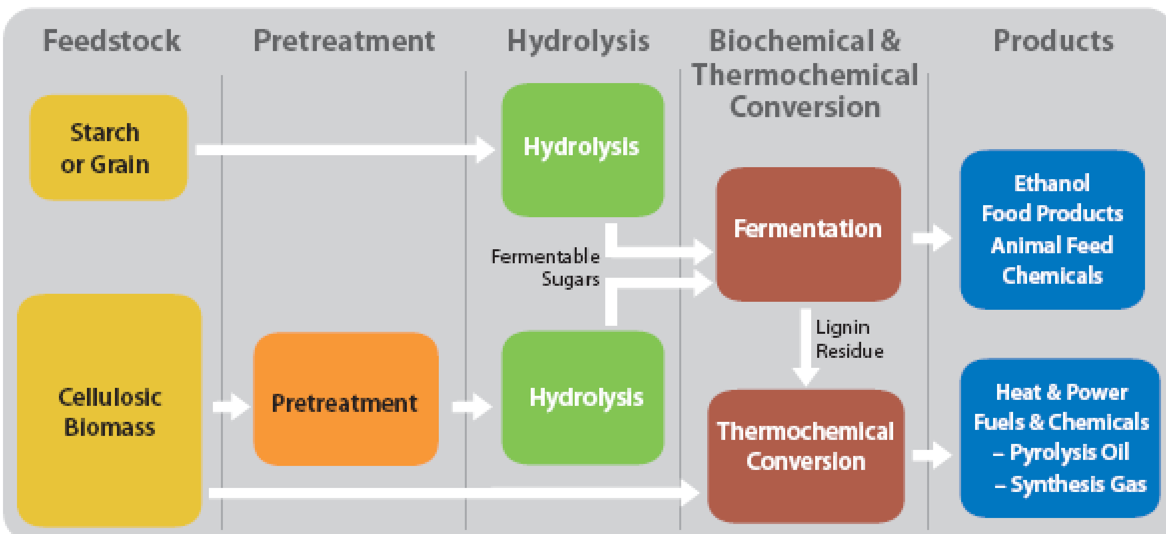


Chart courtesy of the National Renewable Energy Laboratory.

The steps in the diagram above may be summarized as follows:

- **Biomass Handling.** Biomass goes through a size-reduction step to make it easier to handle and to make the ethanol production process more efficient. For example, agricultural residues go through a grinding process and wood goes through a chipping process to achieve a uniform particle size.

- **Biomass Pretreatment.** In this step, the hemicellulose fraction of the biomass is broken down into simple sugars. A chemical reaction called hydrolysis occurs when dilute sulfuric acid is mixed with the biomass

[redacted]

feedstock. In this hydrolysis reaction, the complex chains of sugars that make up the hemicellulose are broken, releasing simple sugar. [redacted] A small portion of the cellulose is also converted to glucose in this step.

- **Enzyme Production.** The cellulase enzymes that are used to hydrolyze the cellulose fraction of the biomass are produced in this step. Alternatively the enzymes might be purchased from commercial enzyme companies.

- **Cellulose Hydrolysis.** In this step, the remaining cellulose is hydrolyzed to glucose. In this enzymatic hydrolysis reaction, cellulase enzymes are used to break the chains of sugars that make up the cellulose, releasing glucose. Cellulose hydrolysis is also called cellulose saccharification because it produces sugars.

- **Glucose Fermentation**. The glucose is converted to ethanol, through a process called fermentation. Fermentation is a series of chemical reactions that convert sugars to ethanol. The fermentation reaction is caused by yeast or bacteria, which feed on the sugars. As the sugars are consumed, ethanol and carbon dioxide are produced.

- [redacted]

- **Ethanol Recovery**. The fermentation product from the glucose and pentose fermentation is called ethanol broth. In this step the ethanol is separated from the other components in the broth. A final dehydration step removes any remaining water from the ethanol.

[redacted]

- **Lignin Utilization.** Lignin and other byproducts of the biomass-to-ethanol process can be used to produce the electricity or steam required for the ethanol production process. Burning lignin actually creates more energy than needed and selling electricity may help the process economics.

7.2.6 Third Party Packaging Facility

It is possible that third party packaging facilities could be utilized to repack the modified yeast from 1000 Kg super sacks into smaller units, such as 20 Kg bags. In the event that such activities do take place the following practices will be followed.

- The facility will have Standard Operating Procedures (SOP) in effect to prevent the cross contamination of other products packed on the same equipment.
- There will be SOP in place to address the accidental release of the modified yeast due to a spill that will prevent the organism from being introduced into the environment without first being subject to a kill step; such as the use of hypochlorite solution to wet the spilled material.
- There will be sufficient engineering controls on the exhaust air.
- There will be SOP in place to protect the workers handling the modified yeast per the product MSDS.

7.3 Worker Exposure Information

The following information is being submitted in accordance with 40 C.F.R. § 725.155(h). For estimates of worker exposure related to the use of the production strain, the Submitter is primarily relying on EPA's 1997 final risk assessment which evaluated potential worker exposures from large-scale, conventional fermentation processes based on information available from eight pre-manufacture notices submitted to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms (Reilly, 1991).

The values EPA selected at that time were based on reasonable worst-case scenarios. As the Submitter expects that the production strain will be used at up to [redacted] and the majority of these will be of the same facility type as EPA has already evaluated, it is respectfully submitted that the conclusions that the agency reached in its 1997 Risk Assessment with respect to worker exposure are relevant and appropriate for evaluating this MCAN.

[redacted]

7.3.1 Maximum Number of Persons Exposed

The microorganisms are intended to be used at sites not under the control by the submitter. The maximum number of persons exposed is provided in Table I below. Worker exposure is minimized by using a highly automated manufacturing process. This process allows for the transfer of raw materials, fermentation broth, and process streams from one step to another with minimal human exposure. Once the fermentation vessel is inoculated, the closed nature of the fermentation process and the automation of the other manufacturing steps result in minimal opportunity for worker exposure.

EPA estimates that a typical site employs less than10 workers/shift and operates 24 hours/day throughout the year (Reilly 1991). Our research found a reference that estimated 28 workers in a ethanol facility that produces 15 million gallons of ethanol per year[7]. Assuming that the 28 workers are split between two shifts, this means that there are 14 workers per shift. In our table below, we have based our estimates of worker activities on 14 workers per shift.

[redacted] Existing biosafety, laboratory, or occupational safety requirements call for employees to be equipped with proper microbiological personal protection equipment when handling live microorganisms which may include a long sleeved lab coat, latex gloves, and safety glasses equipped with side shields.

Table 2 – Worker Exposure

	# of workers per activity	Protective Equipment / Controls	MAXIMUM FREQUENCY OF ACTIVITY (DAYS)	WORK DAY DURATION (Hrs)	Maximum Duration Exposed Hrs /day	Maximum Duration Exposed Days/Yr.
Transportation [redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

7 U.S. Dry-mill Ethanol Industry, National Biobased Products & Bioenergy Coordination Office, U.S. Dept. of Energy

[redacted]

	# of workers per activity	Protective Equipment / Controls	MAXIMUM FREQUENCY OF ACTIVITY (DAYS)	WORK DAY DURATION (Hrs)	Maximum Duration Exposed Hrs /day	Maximum Duration Exposed Days/Yr.
Fermentation Sampling	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

7.3.2 Transportation and Receiving

Commercial volumes, container types, and physical state of the product are described in Section 5 above. Shipment of yeast from the submitter's facilities to the repackaging facility and the ethanol facility is expected to occur via ground transportation in sealed containers in full compliance with DOT regulations. Transfer from the seed propagation laboratory to the fermentor will be via surface roadways within the submitters' facility.

Once the production organism is delivered to the ethanol fermentation facility the strain is intended to be used in a fermentation system, and is not intended to be dispersed into the environment.

It is anticipated that three transport personnel will be used with approximately 17 hours duration exposed per year.

7.3.3 Seed Propagation at the Submitter's Facility

Maintenance and storage of the seed stock will be at the fermentation facility laboratory under the supervision of a laboratory microbiologist. It is anticipated that one employee will be responsible for maintenance and storage of seed stock.

In the seed propagation laboratory, dextrose, nutrients, and water are placed into a laboratory incubator and prior to starting the seed stock all the equipment are sterilized in place at> 120° C with indirect steam The MCAN microorganism working stock culture is thawed at room temperature in a biosafety hood and is aseptically drawn into a pipette. The cells are transferred from the pipette via a sterile needle through a rubber septum into the reactor. The cells are then propagated. When cell biomass reaches the appropriate level, which ranges from 6-10 hours, the incubator vessel containing the cells is transferred to fermentation facility. It is estimated when operating at full capacity that one employee will perform the seed propagation procedure.

[redacted]

When empty, the incubator vessel will undergo manual cleaning with high temperature water. The incubator vessels are rinsed with water and transferred to waste water treatment (WWT). It is estimated when operating at full capacity that one employee will perform this manual cleaning procedure. Propagation will be supervised by one employee.

Personnel protective equipment may include, but is not limited to, safety glasses, full face shield, gloves, full-body apron, lab coat, or plant uniform all of which should be fully disposable and able to enter an autoclave. It is anticipated that exposure to an MCAN microorganism in its viable state will be limited to the following circumstances: sampling, inoculation, manipulation, cleaning in place procedures and storage tank condensation.

Exposure by three plant production technicians to the MCAN microorganism will total approximately [redacted]. This total exposure time is distributed in the following manner; sampling [redacted], inoculations [redacted] cleaning in place [redacted] and storage tank condensation [redacted]

7.3.4 Growth, Harvesting and Packaging at the Submitter's Facility

As noted in sec. 7.1 and figure #3, the submitter will be growing, harvesting and packaging the modified yeast. Worker exposure is addressed in sec. 7.1.5.

7.3.5 Propagation at a Bioethanol Facility

It is acknowledged that ethanol fermentation includes manufacturing of yeast at the seed propagation stage. The yeast are removed from shipment containers and placed into the propagation vessel containing water and nutrients. The broth is agitated for several hours to allow the active dry modified yeast to rehydrate. During this time some growth of the organism ensues, resulting in an increase in organism number by 2 to 4 fold. It is estimated when operating at full capacity that one employee will perform the seed propagation procedure.

Personnel protective equipment may include, but is not limited to, safety glasses, full face shield, gloves, full-body apron, lab coat, or plant uniform all of which should be fully disposable and able to enter an autoclave. It is anticipated that exposure to an MCAN microorganism in its viable state will be limited to the following circumstances: sampling, inoculation, manipulation, cleaning in place procedures and propagation tank condensation.

Exposure by three plant production workers to the MCAN microorganism will total approximately 10 man hours per week. This total exposure time is distributed in the following manner; sampling 0.5 man hours per week, inoculations 4.0 man hours per week, cleaning in place 5.0 man hours per week and storage tank condensation 0.5 man hours per week.

7.3.6 Inoculation

Transfer of the yeast out of the propagation vessels into the ethanol fermentor will be accomplished via hard pipes. Accidental spills will be collected by the production facility's waste sump collection system which is connected via transfer piping to the facility's waste water treatment system or in some facilities to the distillation column feed tank. We anticipate that transfer and inoculation will require two employees.

7.3.7 Fermentation

During fermentation processes worker exposure is possible in the following scenarios:

- Quality control sampling during fermentation
- Harvesting and packaging; and
- Processing and decontamination procedures

Quality control sampling during fermentation is to facilitate process understanding. Small quantities of yeast or active production fermentation broth may be collected in sealed containers by personnel wearing personal protective equipment and transported to the fermentation site's microbiology laboratory for the purpose of running experimental controls.

Yeast growth and sugar conversion to ethanol occur in the production fermentor, which is a closed vessel with an external jacket, air supply, agitators, and sparger. According to [redacted] (see Section 7.2.2 of MCAN) the average fermentor holds 500,000 gallons of ethanol per batch. Average number of fermentors per facility is 4, which can produce approximately 50,000,000 gallons of ethanol or 100 batches each year. Alcohol concentrations for biomass ethanol typical ranges from [redacted]l content, which is a lethal condition for yeast[8]. The ethanol is removed from the fermentation broth through a distillation process. The solid particles, which contain the yeast cells can be removed via centrifugation or filtration or sent to the distillation pot. If the yeast is to be recovered the broth is transferred to the trough of a rotary drum filter or through a centrifuge and to a filter press which further concentrates the solids. The expected efficiency of solids removal for the rotary drum filter is 99% (Reilly 1991). Then an ultrafiltration system is expected to be used to further concentrate the product, in which the liquid leaving the ultrafiltration system is assumed to be free of viable microorganisms (Reilly 1991).

EPA's 1997 exposure assessment drew from NIOSH airborne sampling data taken from several fermentation facilities in the enzyme industry for processes considered typical of fermentation process technology. NIOSH took area samples in locations where the potential for worker exposure was considered to be potentially greatest, *i.e.,* near the fermenter, the seed fermenter, sampling ports, and separation processes (either filter press or rotary drum filter). The workers with the highest potential average exposures at the three facilities visited were those involved in air sampling. Area samples near the sampling port revealed average airborne concentrations ranging from 350 to 648 cfu/m^3. The Submitter is relying on this figure because no other personal sampling data have been provided by customers or are otherwise publicly available.

The Submitter is a yeast manufacturer and not an ethanol producer and such monitoring is not required by OSHA. More to the point, however, there are no known process or equipment changes to the conditions at ethanol facilities that were reviewed by EPA that would cause worst case area sampling data to be inappropriate for use at this time. EPA assumed that 20 total samples per day are drawn and that each sample takes up to 5 minutes to collect, the duration of exposure for a single worker will be about 1.5 hours/day. We assumed the 20 total samples are split between 3-5 sampling events throughout the day with approximately 4-7 samples taken during each sampling event. Assuming that the concentration of microorganisms in the worker's breathing zone is equivalent to the levels found in the area sampling, EPA has estimated that worst-case daily inhalation exposure is estimated to range up estimate to 650 to 1200 cfu/day (Reilly 1991). Although this reference did not provide any calculations to demonstrate how they arrived at the worst case inhalation exposure of650 to 1200 cfu/day based on a

8 Stanley et. al. 2010

[redacted]

1.5 hour exposure period and average airborne concentrations of yeast ranging from 350 to 648 cfu/m^3. We believe those numbers were calculated using a reasonable maximum exposure (RME) rate of30 m3/day average inhalation rate for an adult human for a 24 hour period. (This figure is based on the default EPA assumption of a I 0 cubic meter per day inhalation rate during an 8 hour period 8 hours is one third of 24 hours; 10 cubic meters is one third of 30 cubic meters). We have provided the following calculations for consideration:

<u>Estimates based on a 350 cfu/ m^3 airborne yeast concentration</u>

(30m^3/day) x (day/ 24 hours) x (1.5 hours) x (350 cfu/ m^3/day) = 656 cfu/day

<u>Estimates based on a 648 cfu/m^3 airborne yeast concentration</u>

(30 m^3/day) x (day/24 hours) x (1.5 hours) x (648 cfu/m^3/day) = 1215 cfu/day

However in March 1991, EPA produced a document that recommended an inhalation rate of 20 m^3/day value.[9] Therefore we have recalculate the 650 to 1200 cfu/day yeast exposure rate from the Reilly paper based on the new inhalation rate of 20 m^3/day value below for consideration as the worst case worker exposure for purposes of this submission:

<u>Estimates based on a 350 cfu/ m^3 airborne yeast concentration</u>

(20 m^3/day) x (day/24 hours) x (1.5 hours) x (350 cfu/m^3/day) = 437 cfu/day

---

[9] Exposure values taken from *Exposure Factors Handbook* (EFH) (EPN600/P-95/002F, 1997) and *Risk Assessment Guidancefor Superfund Volume I. Human Health Evaluation Manual (Part A)* (EPA/540/1-89/002, Interim Final, December 1989).

[redacted]

Estimates based on a 648 cfu/m^3 airborne yeast concentration

(20 m^3/day) x (day/24 hours) x (1.5 hours) x (648 cfu/m^3/day) = 810 cfu/day

Therefore based on a inhalation rate of 20 m^3/day the worst case inhalation exposure estimate ranges from 437 to 810 cfu/day. Based on literature review, the modifications do not change the BSL 1 designation for the notified organism. Therefore, we maintain that appropriate environmental release controls should be no greater than that required for any other BS level 1 organism.

7.3.8 Distillation

The ethanol fermentation product is sent to vacuum distillation in which the vapor contains ethanol, water, and other trace volatile organic compounds. The bottoms contain the heavy components such as any remaining cell mass and feedstock residue. The modified yeast is killed during this step of the process.(see **Annexes 7 and 8**) We estimate two workers during the distillation process.

7.3.9 Vessel Clean Out

When empty, the fermentation vessel will undergo cleaning with high temperature water. Worker exposure is expected to be minimal for this task because the workers are not expected to come into direct exposure with the cleaning water, the organism will be inactivated by the water temperature, and workers do not otherwise enter the fermentation vessel or come into direct contact with the viable organism. The vessels are rinsed with water and transferred to waste water treatment (WWT). It is estimated when operating at full capacity that two workers will supervise the cleaning procedure.

[redacted]

8 Environmental Release (sites not controlled by the submitter)

[redacted] to the modified strain does not alter the conditions required for, and conditions that limit or enhance, environmental release, survival, growth and replication. The potential for horizontal gene transfer of [redacted] to occur between eukaryotic cells to prokaryotic cells is low due to the rapid degradation of DNA in the environment, low percentage of competent bacteria that will be naturally present in the environment and low transformation efficiency of competent bacteria (Fink & Moran, 2005). Further considering the lethal effect of the ethanol distillation process on cell viability, it is expected that the dissemination of the notified strain to surrounding areas will be minimal to short distances and limited periods of time. There is no intentional introduction of the organism to the environment. As noted in the survivability study the modified strains have no advantage over the wild-type; Annexes 4 and 5.

The EPA 1997 Final Risk Assessment for *S. cerevisiae* concludes (p. II) that: *"Releases of this microorganism to the environment through fermentation uses would not pose any significant ecological hazards, because this microorganism is ubiquitous in the environment and it is not pathogenic to animals or plants."* Based on a literature review, the modifications do not change the BSL **1** designation for the notified organism. Therefore, we maintain that appropriate environmental release controls should be no greater than that required for any other BS level **1** organism.

NIH Guidelines, Appendix C-III, provides that for large-scale fermentation, physical containment conditions need be no greater than those for the host organism, unmodified by recombinant DNA techniques. In addition, Appendix G-Il-A of the Guidelines specifies the following expected performance criteria for BSL 1:

- **Appendix G-II-A-1-b**. Work surfaces in the laboratory are decontaminated once a day and after any spill of viable material.

- **Appendix G-11-A-1-c**. All contaminated liquid or solid wastes are decontaminated before disposal.

- **Appendix G-11-A-1-d**. Mechanical pipetting devices are used; mouth pipetting is prohibited.

[redacted]

- **Appendix G-11-A-1-e**. Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Food may be stored in cabinets or refrigerators designated and used for this purpose only.

- **Appendix G-II-AC1-f**. Persons wash their hands: (i) after they handle materials involving organisms containing recombinant DNA molecules and animals, and (ii) before exiting the laboratory.

- **Appendix G-II-A-1-g**. All procedures are performed carefully to minimize the creation of aerosols.

- **Appendix G-11-A-1-h**. In the interest of good personal hygiene, facilities (e.g., hand washing sink, shower, changing room) and protective clothing (e.g., uniforms, laboratory coats) shall be provided that are appropriate for the risk of exposure to viable organisms containing recombinant DNA molecules.

- **Appendix G-II-A-2-a**. Contaminated materials that are to be decontaminated at a site away from the laboratory are placed in a durable leak-proof container, which is closed before being removed from the laboratory.

- **Appendix G-11-A-2-b**. An insect and rodent control program is in effect.

- **Appendix G-II-A-3-a**. Special containment equipment is generally not required for manipulations of agents assigned to BL I.

The following information is being submitted in accordance with 40 C.F.R. § 725.155(h).

8.1 Air Release Estimates

EPA has characterized air emission sources in ethanol fermentation facilities to include fermentor vents, openings, seals, and fittings, emergency relief valves, samples operations, rotary drum filters, and storage tank vents. According to EPA, airborne emissions of microorganisms from the fermentor are estimated to range from 7.8 x $I0^2$ to 1.9 x $I0^5$ cfu/m2/sec. Based on these numbers, EPA estimated that a large scale fermentor (considered to be 70,000 gallons at the time of EPA's review in 1991) with minimally contained air emissions of fungi will result in emissions of 2x 10^8 to I x $I0^{11}$

[redacted]

cfu/day. Rotary drum filters are also a source of air emissions, on the order of an additional 250 cfu/day (Reilly 1991).

For purposes of our analysis, we assumed a commercial scale fermentor size of 500,000 gallons of ethanol based on informal discussions with industry experts rather than the 70,000 liter capacity originally modeled by EPA. Further, a reasonable average aeration rate for a 500,000 gallon fermentation vessel based on discussions with industry experts is 3,700 scfm. The cell density is approximately 0.06 g/ml in the fermentation broth based on an expected fermentation concentration of 3.0×10^8 cfu/mL and an estimated 5×10^9 cells per gram of yeast (see Section 7). Assuming the density of water of I g/ml, results in an estimated concentration of approximately. 0.06 g of cells per g of water or 0.06lb. of cells per lb. of water. Assuming that every lbs. of air will contain approximately 0.055 lbs. of water, then each pound of air in a fermentation vessel will contain up to 0.055*0.06 = 0.0033 lbs./hr of cells. The standard density of air is approximately 0.075 lbs./cf, thus the calculated number of cells in the headspace of a fermentor is:

1 scfm = 0.075 lbs./min= 4.5 lb./hour of air

1 scfm = 4.5 lb./hr * 0.0033 = 0.01485 lb. cell per hour

An air flow of 3700 scfm yields 54.95 Lbs./hr cells exhausted (3700 scfm x 0.01485 lbs. cell per hour)

2log reduction (engineering controls)-= (54.95 lbs./hour)/100 = 0.54951bs/hour

According to the submitter, many modern ethanol facilities have sufficient air emission control systems to prevent release of viable yeast during the fermentation process. Nonetheless we propose as a worst case scenario a 2 log reduction from engineering controls, resulted in the above estimate of cells released through air emissions at 0.5495 lbs/hr.

We have converted EPA's 1991 estimates to lbs./hour for comparison with our estimate. At that time, EPA estimated airborne emissions from the fermentor to be approximately 2×10^8 to1×10^{11} cfu/day, and EPA assumes that the typical ethanol facility operates 24 hours/day. With respect to the control of air emissions, EPA assumed a 99% filtration efficiency under normal operating conditions to arrive at these numbers, which is the same as a 2 log reduction. Converting these figures yields the following values for comparison:

(2×10^8 cfu/day) x (I gram /5×10^9 cfu) x (0.002204lb/gram) x (day/24 hours)=

[redacted]

3.6×10^{-6} lbs/hr

$(1 \times 10^{11}$ cfu/day) x (I gram /5×10^{9} cfu) x (0.002204lb/gram) x (day/24 hours)=

1.8×10^{-3} lbs/hr

Therefore EPA gives a worst case estimate of 0.0018 lbs/hr of yeast emission from a 70,000 L fermentor, and our current calculations estimate a 0.5495 lbs/hr of yeast emissions for a 500,000 gallon (I,892,706 L) fermentor. Given the larger size of our fermentation vessel, it is not unreasonable to have a higher yeast emission rate calculations compared to EPA's 70,000 L fermentor.

However, EPA in its final decision to designate *Saccharomyces cerevisiae* as eligible for the tiered exemptions from pre-market approval noted in section IV. Public Comment…;

'…Even under a worst case scenario of an uncontrolled release, as evaluated in the accompanying risk assessment, the number of viable microorganisms aerosolized with the fermentor exhaust gases would still be low, and therefore, the risk would remain low. Moreover, the use of a criterion requiring controls to minimize microbial numbers released through aerosolization at õ 725.422, as compared to the worst case scenario of an uncontrolled release, would result in lesser exposure, and therefore, lower risk than under the uncontrolled release scenario……Therefore, upon re-evaluation, the Agency decided that language requiring minimization of microbial concentrations in aerosols could be substituted for the requirement of the 2-log reduction performance criterion without affecting the no unreasonable risk finding necessary for a 5(h)(4) exemption under TSCA. The potentially increased exposure to this organism from the modification of the containment criteria from the proposed 2-log reduction to minimizing microbial numbers in exhaust gases does not change the risk of using this microorganism for fermentation. Therefore, EPA has revised õ 725.422(e) to read: "Use features known to be effective in minimizing viable microbial populations in aerosols and exhaust gases released from the structure, and document use of such features"

The submitter believes that users of the modified yeast will employ engineering controls to minimize the viable organism population in the aerosols and exhaust gases released from the structure.

[redacted]

8.1.1 Evaluation of Air Release Estimates

We think a 2 log reduction is achievable given the regulation of emissions from these facilities under the Clean Air Act. As indicated in the Nebraska Dept. of Environmental Quality Report of 2008,**(Nebraska 2008)** the EPA air program office and delegated state authorities are working to control air emissions from ethanol production facilities. It is respectfully submitted that these efforts are sufficient to address emissions associated with the use of the production strain notified in this MCAN and that today's engineering controls are assumed to achieve an at least a 2 log reduction in the expected level of release of the microorganism relative to the microbial numbers in the fermentor gases in the headspace and in comparison to estimated off-gases without this control in place. Because the cells are large most are expected to remain with the liquid stream.

In 2002, EPA began investigating a suspected pattern of noncompliance with the Prevention of Serious Deterioration / New Source Review (PSD/NSR) requirements of the Clean Air Act (CAA) within the ethanol industry. The Clean Air Act's NSR program requires a source to install pollution controls and undertake other pre-construction obligations to control air pollution emissions. Subsequent investigations of several companies in the ethanol industry found them to be in violation for failure to obtain either PSD or minor source permits for new construction and/or modifications made at twelve facilities in Minnesota. Agreements announced on October 2, 2002 required twelve plants to install air pollution control equipment to greatly reduce air emissions. All the companies were required to install the Best Available Control Technology (BACT) and obtain appropriate permits from the state of Minnesota. Under the settlements, the plants were to install thermal oxidizers that reduced VOC emissions by 95 percent from the feed dryers and that met new, more restrictive emission limits for NOx, PM, CO and hazardous air pollutants. In addition to emission control requirements valued at about $2 million per plant, each facility was required to pay a civil penalty ranging from $29,000- $39,000.[10]

In addition, EPA reached a settlement with Archer Daniels Midland {ADM) encompassing 52 plants in 16 states. The settlement is the result of a joint federal and state enforcement effort with 14 states and counties signing onto the consent decree. Under the settlement, ADM' s improvements at plants nationwide were projected to result in a reduction of at least 63,000 tons of air pollution a year.

A similar settlement was reached in September 2005 with Cargill, the second largest competitor in this grain processing industry sector, which was projected to result in

[10] Ethanol 2000 CAA Ethanol Settlement | Enforcement | US EPA

[redacted]

emission reductions at 27 plants in 12 states with actual reductions of about 25,000 tons per year and reductions in permitted emissions of 40,000 tons per year.

EPA considered a 2 log reduction target as appropriately protective under the Tier I exemption for modified S cerevisiae. EPA states in its Rule on Microbial Products of Biotechnology: Summary of the Public's Comments and the Agency's Response that "[i]n the proposal EPA indicated that a 2 log reduction in viable microorganisms per cubic foot of air between the headspace and the actual vent port was the appropriate standard [for the Tier I exemption]."and the agency characterized its position further as follows[11]:

> *EPA believes that it should allow some flexibility in the type of features manufacturers employ to minimize microbial releases as aerosols. A variety of ferment or equipment or features are commonly used by the industry such as demisters, wet scrubbers, cyclone separators, coalescing filters, and HEPA filters. These types of equipment reduce the number of microorganisms vented through exhaust gases from the fermentor. Moreover, as stated in the preamble (59 FR 45549), even if microorganisms are exhausted from the fermentor, their survival is likely to be limited due to the stress conditions of aerosolization, including shear forces, desiccation, and UV light exposure. Given the comments received on the feasibility of this requirement and the variety of methods used by PMN submitters to reduce microbial numbers in aerosols, EPA believes that a specific numerical performance standard is less appropriate for inactivation of aerosols than it is for inactivation of liquid and solid wastes. EPA agrees with commenters who asserted that the majority of microorganisms potentially released from the fermentation facility would be found in the liquid and solid wastes.*

Further, in its 1997 Final Risk Assessment for *S. cerevisiae* (see EPA 1997) with respect to the use of engineering controls, EPA reviewed information submitted on physical containment and control technologies in the PMNs it had received for intergeneric microorganisms between 1986 and 1995. The following finding is relevant to this assessment:

---

[11] Federal Register, Volume 62 Issue 70 (Friday, April 11, 1997)17910-17958

[redacted]

Examination of these PMNs revealed that the number of microorganisms potentially released through fermenter exhaust gases is negligible compared to the number contained in the liquid and solid waste streams. Even under a worst case scenario of an uncontrolled release, as evaluated in the accompanying risk assessment, the number of viable microorganisms aerosolized with the fermenter exhaust gases would still be low, and therefore, the risk would remain low. Moreover, the use of a criterion requiring controls to minimize microbial numbers released through aerosolization at§ 725.422, as compared to the worst case scenario of an uncontrolled release, would result in lesser exposure, and therefore, lower risk than under the uncontrolled release scenario. Uncontrolled releases are not standard industry practice because there are a number of economic considerations driving the control of exhaust gases such as maintaining proper molarity of the fermentation broth by the use of a vapor recovery system, maintaining sterility, and preventing release of microorganisms for proprietary reasons. Therefore, upon re-evaluation, the Agency decided that language requiring minimization of microbial concentrations in aerosols could be substituted for the requirement of the 2-log reduction performance criterion without affecting the no unreasonable risk finding necessary for a 5(h)(4) exemption under TSCA. The potentially increased exposure to this organism from the modification of the containment criteria from the proposed 2-log reduction to minimizing microbial numbers in exhaust gases does not change the risk of using this microorganism for fermentation.

[redacted]

8.2 Water Release Estimate

The Submitter is relying on an estimated concentration of3.0 x 108 yeast cells/mL in a 500,000 gallon fermentor. Therefore the submitter's estimates will be based on a larger fermentation vessel with a lower cell density concentration compared to EPA's estimates. The total number of yeast cells in a 500,000 gallon fermentor with an estimated concentration of 3.0 x I 08 yeast cells/mL is calculated as follows:

500,000 gallons x (3.785 L/1 gallon) x (IOOO mL/1 L) x 3.0 x 10^8 cells/mL = 5.0 x 10^{17} cells per fermentation batch in the fermentor.

A 0.4% residual of cells is assumed in a fermenter following fermentation is used for purposes of the following analysis. This figure was derived from EPA's 1991 characterization of sources of water releases to include steam condensate, streams from the sterilization or cleaning of the fermentor vessel and filters, disposal of samples, residues in the rotary filter trough, and cleaning wastes from the ultrafiltration membrane. (Reilly 1991) From EPA's analysis, EPA cites the average assize to be70,000 liters with a cell concentration for fungi to be $I0^9$ cfu/mL. Therefore we can calculate the total number of cells in fermentor to be:

70,000L x (1000mLIIL) x 10^9 cell (mL) = 7 x 10^{16} cells in a fermentor.

In this same document, EPA also estimates an average 4 day fermentation period per batch and an estimated release to water to be about 7 x 10^{12} cfu/day. Therefore over a period of 4 days, we calculate the total number of released cells as follows:

(7 x 10^{12} cfu/day) x (4 days/batch) = 2.8 x 10^{13} cfu/batch.

Given that each fermentor contains 7 x 10^{16} cells and 2.8 x 10^{13} cfu are released over the 4 day period, we can calculate the percentage of cells release as follows:

[redacted]

$(2.8 \times 10^{13}$ cfu/batch) 17×10^{16} cells = 0.0004 or 0.04%

Therefore based on information provided in EPA's document (Reilly 1991) we estimate that 0.04% of the cells are flushed into the waste water stream from a 70,000 L fermentor. As the submitter's calculations (see above) of the number of cells in a 500,000 gallon fermentor to be 5.0×10^{17} cells per fermentation batch in the fermentor, 0.04% of the cells would be calculated as follows:

$(5.0 \times 10^{17}$ cells)* 0.0004 = 2.0×10^{14} cells/batch released into waste water

Therefore our estimates for cell mass residue left in the fermentation vessel that can be flushed to POTW during the cleaning of the fermentation vessel into the waste water are expected to be higher due to the increased number of total cells in our larger fermentor (500,000 gallons).

We are assuming that the waste water does not contain live yeast cells after the yeast have been subjected to lethal temperature treatment during distillation, and physical removal from the liquid to be used in distillers grain. (www.distillersgrains.org/grains) According to informal discussions with the ethanol facility management company, [redacted], distillation temperatures after fermentation reach a range of 71-82°C. Vat pasteurization conditions, which kill yeast cells, are achieved at 65° C for 30 minutes or High Temperature Short Time pasteurization (HTST) at 72° C for 15 seconds.[12] Therefore the 2.0×10^{14} cells yeast that may be present in the waste water after each fermentation batch are assumed to be inactivated. Liquid from these sources are sent to a kill tank prior to discharge via an onsite waste water treatment or publicly-owned treatment works and approximately 50%, which was selected as a reasonable average assumption based on discussions with industry experts, of the water extracted from the distillation process will be recycled to process the feedstock for the next round of ethanol production.

In some newer facilities it is possible to capture and reuse the CIP water. In these facilities the CIP system is maintained at 200 °F which would inactivate the modified yeast that was washed from the equipment. On those occasions when the CIP system is refreshed with new water and caustic, the used solution is utilized for pH adjustment or

[12] Idfa_org_pasteurization

sent to the distillation system prior to the solids being disposed of as a component of the boiler fuel as previously mentioned.

8.3 Solid Waste

The agency's review concluded that solid waste is expected from disposal of the filter cake, which is typically sent to landfill or spread onto land. EPA estimates that these solid releases are expected to contain inactivated cells on the order of about 7 x I 0^{15} cfu/day.

However, the submitter respectfully submits that there is minimal to no solid waste containing the yeast cells, which are inactive, after distillation as they are expected to be used as an economically valuable component of distillers grain (www.distillersgrains.org/grains/) or used as fuel for the facility's boilers. In the rare case that the modified yeast goes past its shelf life, the product will either be returned to the submitter or undergo caustic treatment to inactive the yeast cells and disposed of according to federal and state regulations.

With respect to the disposal of empty containers after the yeast have been added to the seed propagation tank or directly into the fermentor, the 20 Kg poly-lined, multi-walled kraft paper bags, poly-coated aluminum laminated bags, fiber drums and 1000 Kg woven polyester bags will disposed of in a sanitary land fill.

The yeast residue in the bags of active dried yeast are not expected to thrive in landfill conditions as yeast require a constant supply of moisture and oxygen. Most landfill conditions are low in moisture and anaerobic (lack of oxygen) due to physical compaction of the solid waste, and therefore do not provide an optimal environment for yeast or other aerobic microbes to thrive.[13]

8.3.1 Procedures for Disposal of Waste Articles

[13] bioreactor brochure.pdf

[redacted]

8.3.1.1 Laboratory Waste

All laboratory solid waste known to have been in contact with active biological materials are disposed of in designated biohazard waste receptacles. Biohazard waste is collected on a regular basis and killed using an autoclave heated to> 120° C. Once deactivated this waste is disposed of via a private garbage handler with normal waste streams following local and state industrial waste regulations.

8.3.1.2 Clothing

Clothing that comes into contact with modified yeast is laundered in commercial detergent by industrial uniform management companies at a temperature sufficient to kill the organism.

8.3.1.3 Equipment

Laboratory equipment, including bench tops are disinfected using 70% isopropyl alcohol or equivalent after coming into contact with an MCAN microorganism. When acceptable, equipment will be placed into an autoclave and heated to > 120° C in order to decontaminate prior to re-use. Production equipment is cleaned in place and is sanitized or sterilized depending upon the facility’s operating practices prior to each production run.

8.3.2 Spills and Emergency Preparedness Measures

Ethanol production facilities will typically be subject to state and federal requirements to have procedures in place that provide appropriate hazard and emergency preparedness measures. Aspects may include the following: emergency classification system, government response, incident command, and evacuation/accountability. On-site emergency procedures will call for containment, deactivation (through use of dilute bleach), proper disposal, and the use of personal protective equipment. In addition, facilities may have trained HazMat Technicians on-site to evaluate and respond to process upsets.

[redacted]

9 Procedures for Terminating the Organism

9.1 Termination of the Modified Yeast at [redacted] Facilities

As noted in sections 7.2.2 corn-based ethanol production and 7.2.5 cellulosic ethanol production, the modified yeast is transferred with the fermentation mash to the distillation process when the conversion of sugars to ethanol is complete. The temperatures utilized in the distillation process are of a sufficient level and duration of exposure to ensure a 6 log reduction in viability of the organism. A more detailed substantiation of the destruction of a predecessor strain of modified yeast is provided in **Annexes 7** with additional secondary references in Alcohol Book chapters 16, 19 and 27.

9.2 Termination Under Exceptional Circumstances

It is generally not necessary, based on the ubiquitous nature of the organism, its lack of pathogenicity, and the nature of the ethanol production process to have termination procedures per se. Cross-contamination incidents will be unlikely due to SOPs, including cleaning of the fermenters before and after use. However, in the event of cross-contamination or for other contamination reasons, termination may be desired. In that event,

1. the fermentation broth and all other liquid in the process would be processed through distillation
2. the distillers' grains and any other biomass would be dried
3. the fermenters and all equipment would undergo CIP rinses; and
4. all water and CIP liquids would usually be treated.

[redacted]

10 Health and Safety Data

As required by 40 C.F.R. §725.160, all testing regarding the health and environmental effects conducted on the microorganism known to the Submitter have been provided with this MCAN. Copies of the studies and references in the Submitter's possession and control have also been provided with this MCAN.

[redacted]

11 Attachments

1) Annex 1- Primers – CBI
2) Annex 1 – Primers – Redacted
3) Annex 2 - Growth test via plating assay-CBI
4) Annex 2 - Growth test via plating assay-Redacted
5) Annex 3 – BLAST search
6) Annex 3 – BLAST search-redacted
7) Annex 4 - Environmental Survival Study [redacted]
8) Annex 4 - Environmental Survival Study [redacted]
9) Annex 5 - Environmental Survival Study [redacted]
10) Annex 5 - Environmental Survival Study [redacted]
11) Annex 6 - Study Results, Exposure of Corn Plants and Grass to [redacted]
12) Annex 6 - Study Results, Exposure of Corn Plants and Grass to [redacted]
13) Annex 7 - Evidence that the modified yeast is inactivated by the distillation process-CBI
14) Annex 7 - Evidence that the modified yeast is inactivated by the distillation process-Redacted
15) Annex 8 - lab procedures – CBI
16) Annex 8 - lab procedures – Redacted
17) Annex 9 – Product data sheet
18) Annex 9 – Product Data Sheet – redacted
19) Annex 10 - References